Metabolomics for studying diabetes and obesity

Rogova, Oksana

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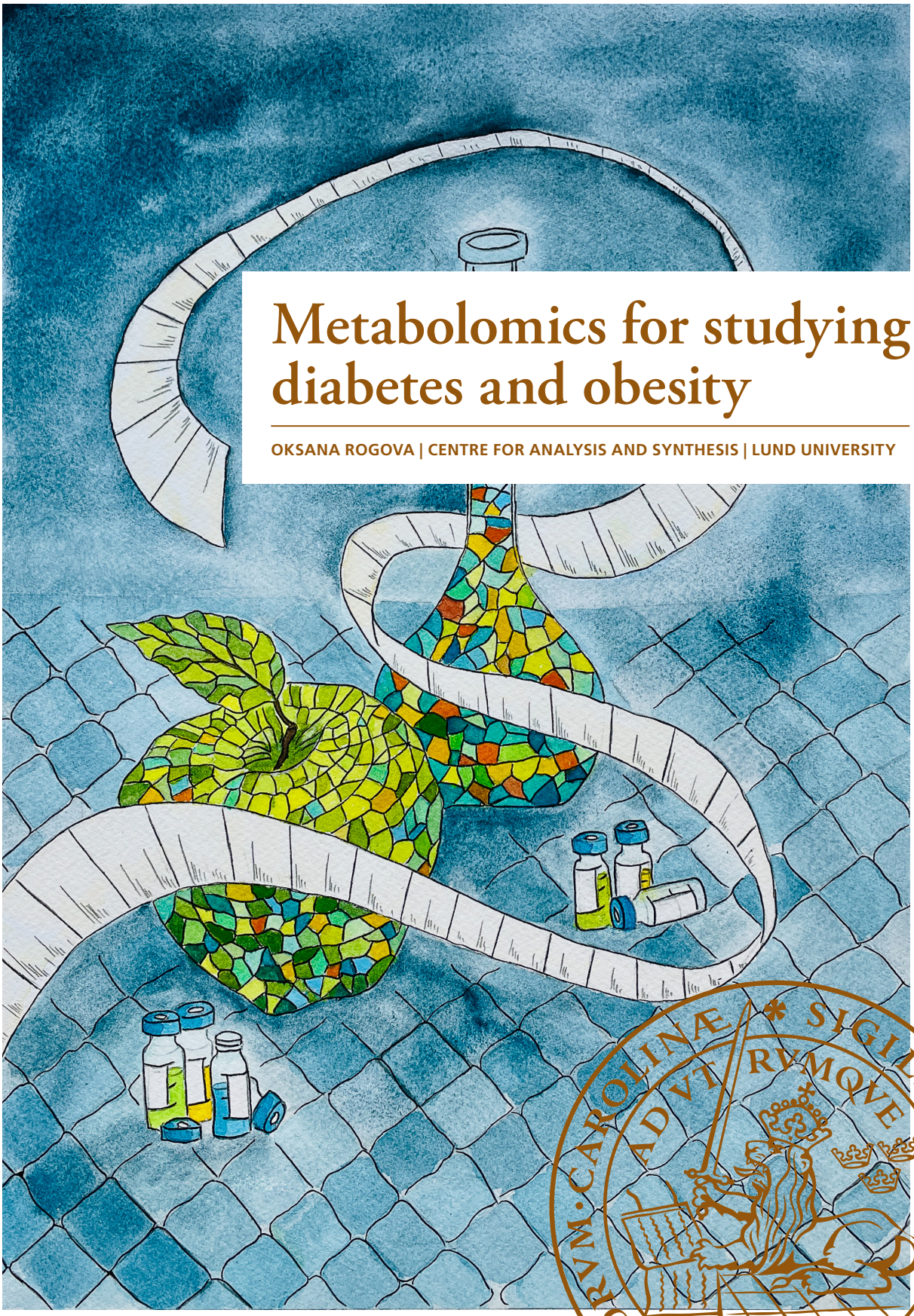

Metabolomics for studying diabetes and obesity

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Oksana Rogova

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract:

Introduction: Obesity continues to be a growing problem and places a significant burden on the healthcare system by increasing the risk of developing various diseases including type 2 diabetes (T2D). T2D, in turn, increases the risk of developing other diseases, such as cardiovascular diseases (CVDs), neuropathy, and nephropathy. Furthermore, obesity and T2D are closely linked to liver diseases, particularly metabolic dysfunction-associated steatotic liver disease (MASLD). Various strategies exist to overcome obesity, including lifestyle modification, food supplements, pharmaceuticals, and more invasive surgical methods. In this thesis, the impact of various obesity prevention strategies on metabolism is assessed using metabolomics and lipidomics.

Methods: I applied liquid and gas chromatography coupled with mass spectrometry to obtain metabolite and lipid profiles, followed by statistical analysis of the resulting data. In Paper I, I evaluated the effect of Roux-en-Y gastric bypass surgery (RYGB) on human metabolism within a few months and a few years post-surgery. In Paper II, I studied the effect of caffeine supplementation on metabolism in the healthy liver, both in vivo and in vitro. In Paper III, I examined the effect of meals enriched in carbohydrates, fats, protein, or fibre on individuals with type 1 and type 2 diabetes, as well as normoglycaemic individuals.

Results and Discussion: In Paper I, I found that the majority of changes in the metabolome and lipidome occurred within two months after RYGB, after which the metabolic profiles began to reverse, moving towards their initial state. In Paper II, I did not find any metabolite or lipid to be significantly altered due to caffeine supplementation in models of the healthy liver, suggesting that the beneficial effect of caffeine may only be found in the diseased liver. In Paper III, I found metabolism to differ between individuals based on their glycaemic status and in response to variations in meal composition. Minor differences were observed in the diabetes status-dependent response to meal variation.

Conclusion: RYGB significantly affected the human metabolome, however, these changes were transient, and within few months after surgery, metabolic profiles began to shift back towards their initial state. The beneficial effect of caffeine supplementation on liver was not observed in models of the healthy liver. The lack of diabetes status-dependent responses to meal variation suggests that diets that are healthy in people without diabetes are also healthy in people with diabetes.

Key words: Metabolomics, lipidomics, diabetes, obesity, chromatography, mass scpectrometry

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Above all, don't fear difficult moments.
The best comes from them.

Rita Levi-Montalcini,
the winner of 1986 Nobel Prize in Physiology.

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Abstract

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A sincere thank you to all of you!

Abbreviations

AC	acyl carnitine
CE	cholesterol ester
CVD	cardiovascular disease
DDA	data-dependent acquisition
DG	diglyceride
DIA	data-independent acquisition
EI	electron ionisation
ESI	electrospray ionisation
FA	fatty acid
FT ICR	Fourier-transform ion cyclotron resonance
GC	gas chromatography
GLP-1	glucagon-like peptide-1
HILIC	hydrophilic interaction liquid chromatography
IS	internal standard
LC	liquid chromatography
LPC	lysophosphatidylcholine
LPC-O	ether-linked lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
MASLD	metabolic dysfunction-associated steatotic liver disease
MRM	multiple reaction monitoring
MS	mass spectrometry
MTBE	methyl tert-butyl ether
NAFLD	non-alcoholic fatty liver disease
NP-LC	normal-phase liquid chromatography

OPLS-EP	orthogonal projections to latent structures-effect projections
PC	phosphatidylcholine
PCA	principal component analysis
PC-O	ether-linked phosphatidylcholine
PE	phosphatidylethanolamine
PE-O	ether-linked phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
QC	quality control
QQQ	triple quadrupole
RP-LC	reversed-phase liquid chromatography
RYGB	Roux-en-Y gastric bypass surgery
SFC	supercritical fluid chromatography
SM	sphingomyelin
T1D	type 1 diabetes
T2D	type 2 diabetes
TCA	tricarboxylic acid cycle
TG	triglyceride
tims	trapped ion mobility spectrometry
TOF	time of flight

List of Papers

I. *Metabolic remission precedes possible weight regain after gastric bypass surgery.*

Oksana Rogova, Katharina Herzog, Mahmoud Al-Majdoub, Michael Miskelly, Andreas Lindqvist, Louise Bennet, Jan Hedenbro, Nils Wierup and Peter Spégel. 2023. Obesity, 31: 2530-42.

II. *Absence of caffeine impact on hepatic metabolism in healthy conditions: Insights from human cross-sectional studies, in vivo and in vitro experiments*

Oksana Rogova, Katharina Herzog, Jasper-Peter Frilund, Klinsmann Carolo dos Santos, Mahmoud Al-Majdoub, Matteo Bruschettini, David Ley, and Peter Spégel

Manuscript

III. *The impact of isocaloric meals with varying macronutrient composition on the metabolome and lipidome of individuals with and without type 1 or type 2 diabetes*

Oksana Rogova, Sergiu-Bogdan Catrina, Neda Rajamand Ekberg, Peter Spégel.

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IV. *Genetic deletion of hormone-sensitive lipase in mice reduces cerebral blood flow but does not aggravate the impact of diet-induced obesity on memory*

Cecilia Skoug, **Oksana Rogova**, Peter Spégel, Cecilia Holm, and João M.N. Duarte. 2024. Journal of Neurochemistry, 168: 781-800.

V. *A randomized trial involving a multifunctional diet reveals systematic lipid remodelling and improvements in cardiometabolic risk factors in middle aged to aged adult.*

Claudia Balderas Arroyo, Maider Greño Ocariz, **Oksana Rogova**, Mahmoud Al-Majdoub, Inger Björk, Juscelino Tovar, and Peter Spégel. 2023. Frontiers in Nutrition, 10.

VI. *EHD2 regulates plasma membrane integrity and downstream insulin receptor signalling events.*

Mathis Neuhaus, Claes Fryklund, Holly Taylor, Andrea Borreguero-Muñoz, Franziska Kopietz, Hamidreza Ardalani, **Oksana Rogova**, Laura Stirrat, Shaun K. Bremner, Peter Spégel, Nia J. Bryant, Gwyn W. Gould, and Karin G Stenkula. 2023. Molecular Biology of the Cell, 34:12

VII. *Using phosphoglucose isomerase-deficient (pgi1Δ) Saccharomyces cerevisiae to map the impact of sugar phosphate levels on d-glucose and d-xylose sensing.*

Borgström, Celina, Viktor C. Persson, **Oksana Rogova**, Karen O. Osiro, Ester Lundberg, Peter Spégel, and Marie Gorwa-Grauslund. 2022. Microbial Cell Factories, 21: 253.

VIII. *Targeted Metabolite Profiling using Gas Chromatography Electron Ionization and Chemical Ionization Mass Spectrometry in Single Reaction Monitoring Mode – A Comparative Study.*

Simon Palmer, **Oksana Rogova**, and Peter Spégel

Manuscript

IX. *Repurposing statins and phenothiazines to treat chemoresistant neuroblastoma*

Katarzyna Radke, Kristina Aaltonen, Erick Andrés Muciño-Olmos, Adriana Mañas, Alexandra Seger, Aleksandra Adamska, Javan Esfandyari, Karin Hansson, **Oksana Rogova**, Daniel J. Mason, Daniel J. O'Donovan, Ian Roberts, Antonia Lock, Jane Brennan, Emma J. Davies, Peter Spégel, Oscar Bedoya Reina, David Brown, Neil T. Thompson, Cesare Spadoni, Daniel Bexell.

Manuscript submitted to the Journal of Clinical Investigation

Author contribution

I. I performed sample derivatisation and GC-MS analysis; processing of raw lipidomics and GC-MS metabolomics data; performed the vast majority of statistical data analysis in R and wrote the first version of the manuscript.

II. I performed processing of raw lipidomics data from *in vivo* experiment; developed a LC-QQQ method for targeted lipid analysis; performed sample preparation of cell samples from *in vitro* experiment and their lipidomics analysis by LC-QQQ followed by raw data processing; performed data analysis in R and wrote the first version of the manuscript.

III. I developed an automated sample preparation workflow on a robotic liquid handling platform for the extraction of polar and lipophilic metabolites and applied it to the samples to get both types of extracts for each sample; performed derivatisation of polar metabolite extracts followed by their GC-MS analysis; performed analysis of lipophilic metabolites with LC-timsTOF followed by processing of raw lipidomics and GC-MS metabolomics data; performed the vast majority of statistical analysis in R and wrote the first draft of the manuscript

IV. I performed sample preparation and lipidomics analysis using the targeted LC-QQQ method.

V. I contributed to the preparation and GC-MS analysis of plasma samples and subsequent metabolomics data processing.

VI. I performed sample preparation and lipidomics analysis using the targeted LC-QQQ method.

VII. I adapted the method for quantification of sugar phosphates in yeast culture, performed sample preparation, analysis by LC-QQQ, raw data processing, statistical data analysis and quantification.

VIII. I contributed to the GC-MS analysis.

IX. I performed sample preparation and lipidomics analysis using LC-timsTOF.

Popular Science Summary

Although humanity has found ways to manage the COVID-19 pandemic, another silent pandemic – obesity – continues to affect people around the world. Obesity is considered a disease because of its harmful effects on health. It increases the risk of developing several diseases, such as heart disease and type 2 diabetes (T2D). T2D, in turn, also negatively affects human health and, over time, may lead to serious damage to blood vessels, nerves, the heart, eyes, and kidneys. T2D and obesity are also linked to a liver disorder called metabolic dysfunction-associated steatotic liver disease (MASLD), where fat builds up in the liver.

One possible explanation for the harmful effects of obesity is based on the theory of lipotoxicity. In the body, excessive energy is stored in the form of fat in adipose tissue. However, when the amount of incoming fat exceeds the storage capacity of the adipose tissue, fat begins to accumulate in other cells in the body, where it can interfere with the normal function of cells.

There are several ways to combat obesity, including lifestyle changes, dietary supplements, medications, and even surgery. This thesis investigates the impact of various obesity treatments on metabolism using two scientific approaches: metabolomics and lipidomics. These approaches are similar to blood tests but allow for the analysis of hundreds of different compounds in a sample. The key difference between metabolomics and lipidomics is that metabolomics focuses on water-soluble compounds, such as amino acids and sugars, while lipidomics targets oil-soluble compounds, such as fats.

In **Paper I**, we studied how the human metabolome – the complete set of small molecules in the body – changes after a weight loss procedure called Roux-en-Y gastric bypass surgery (RYGB). In this surgery, the stomach size is reduced, and the upper part of the small intestine is bypassed, which limits the amount of food people can eat and also limits food absorption. After RYGB, people can lose up to 30% of their body weight within a year. It is also known that people with T2D experience improvements in their blood glucose levels shortly after surgery. However, not everyone maintains these improvements, and some people regain weight and experience a return of T2D over time. In our study, we analysed blood samples obtained before RYGB and after two months, one year, and five years after the surgery. We found that the metabolite and lipid levels significantly changed after surgery but started to return to their original state two months after surgery. This

may suggest the body's adaptation to the surgery, but it can also be early signs of future weight regain.

In **Paper II**, we examined the impact of caffeine on fat accumulation in the liver. Coffee and caffeine are suggested to be good for health, including lowering the risk of T2D and liver diseases, but the exact mechanism of this is not well understood. Some studies suggest that caffeine helps reduce fat build-up in the liver, but most of these studies have been done on unhealthy liver models. Our study focused on healthy liver models and found that caffeine, at normal concentrations, did not have a significant effect on fat accumulation in the liver.

In **Paper III**, we studied how the human metabolome responds to four different meals equal in calories but enriched in different macronutrients: carbohydrates, fats, protein, or fibre. The study was conducted in three groups of participants: people with type 1 diabetes and T2D, and a healthy control group. We found that the metabolite and lipid profiles of the T2D group were the most different from the other two groups. Additionally, the levels of several metabolites and lipids varied depending on the type of macronutrient in the meal. However, we did not find enough evidence to show that people in the three groups responded differently to the same meal.

1. Introduction and Background

1.1. What is metabolomics and lipidomics?

Metabolomics is the large-scale study of the metabolome, which is the combination of all small molecules – *metabolites* – present in a cell, tissue, biofluid, or organism [1, 2]. *Lipidomics* represents a subset of metabolomics and aims to study lipids present in a biological system [3]. Often, the term "metabolomics" is used to describe the polar part of the metabolome, which includes compounds such as amino acids, sugars, and carboxylic acids, while "lipidomics" refers to the lipophilic part, encompassing several lipid classes. The terminology outlined above will be used consistently throughout this thesis.

Metabolomics and lipidomics belong to the omics family of disciplines, aiming to characterise all the entities within a certain biological subset. For instance, genomics aims to characterise all genes, transcriptomics – all RNAs, proteomics – all proteins, metabolomics – all metabolites in a particular biological system (**Figure 1**) [1].

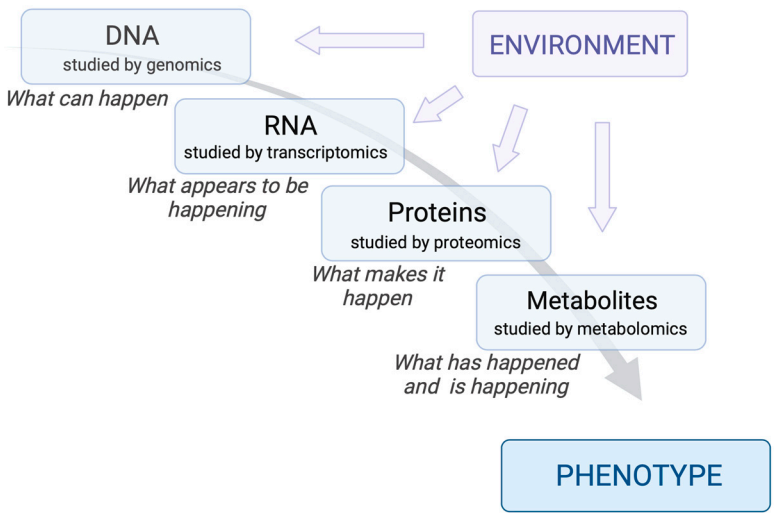


Figure 1. Illustration of different omics fields and the transmission of information from genes via RNA and proteins to metabolome, which together provide information about an organism's phenotype. | Redrawn with adaptation from Dettmer, K. et al. [1]. Copyright © 2006, John Wiley and Sons.

The main advantage of metabolomics is that it provides a snapshot of an organism's state under certain conditions at a specific time. This allows for the evaluation of the *phenotype* – a unique combination of various characteristics defining a particular biological system as a result of the combination of *endogenous* biological processes in the organism (predefined by genome) under the influence of *external* environmental processes [4]. Environmental factors in human research can be determined as very broad range of factors, such as diet, medications, ecological factors and compounds produced by gut microbiota. By contrast, endogenous factors, apart from genetically predefined ones, also include pathological processes ongoing in the body, such as obesity and diabetes in humans, which are the focus of this thesis.

Analysis of an organism's metabolome/lipidome is challenging due to its high complexity. The focus of this thesis is on the human metabolome, which is exceptionally diverse and estimated to consist of >100 000 molecules, among which researches are able to measure about 8000 molecules currently [2, 5-7]. Additionally, human metabolites have high structural heterogeneity and cannot be measured using only one method (in contrast to genomics and to some extent proteomics) and usually require sample preparation and analysis on several platforms. The metabolomics workflow usually consists of several steps, such as sample collection, sample preparation, sample analysis, and evaluation of the obtained data [2]. More details about metabolomics sample preparation and analysis are given in the **Methods** section of this thesis.

Two main approaches are used to detect metabolites: *nuclear magnetic resonance* (NMR) and *mass spectrometry* (MS) [8]. NMR-based metabolomics is non-destructive, allows simultaneous determination of all measurable metabolites present in the sample, and can be utilised in dynamic and *in vivo* studies [9]. NMR-based metabolomics has the advantages of high reproducibility, the ability to quantify metabolites in a wide dynamic range (wide range of concentrations), high sample throughput, the possibility to identify isomers, and determine the structure of unknown metabolites, especially when combined with modern software tools for automated metabolite identification. NMR-based metabolomics can be beneficial for the measurement of compounds that are difficult to ionise or require derivatisation prior to MS analysis [9]. However, NMR metabolomics has its limitations, such as high detection limits, low resolving power, and usually requires larger sample volumes compared to MS-based techniques [10, 11].

MS is currently the most widely used detection technique in metabolomics due to its main advantages: it allows measurements of metabolites present at very low concentrations, and can be easily coupled with complementary separation techniques, such as chromatography and ion mobility, which significantly extend the number of detected compounds [10, 12]. At the same time, the disadvantage of MS-based metabolomics is that it often requires more complicated sample preparation methods to reduce sample complexity, may need sample derivatisation

and encounters difficulties in analysing compounds with poor ionisation yields [10], [9].

In my thesis, I focused on metabolomics and lipidomics analysis of human plasma and used chromatography and MS-based techniques. This approach usually consists of sample preparation, separation using gas or liquid chromatography, and detection with mass spectrometry [12, 13].

1.2. Diabetes, obesity, and metabolic dysfunction-associated steatotic liver disease

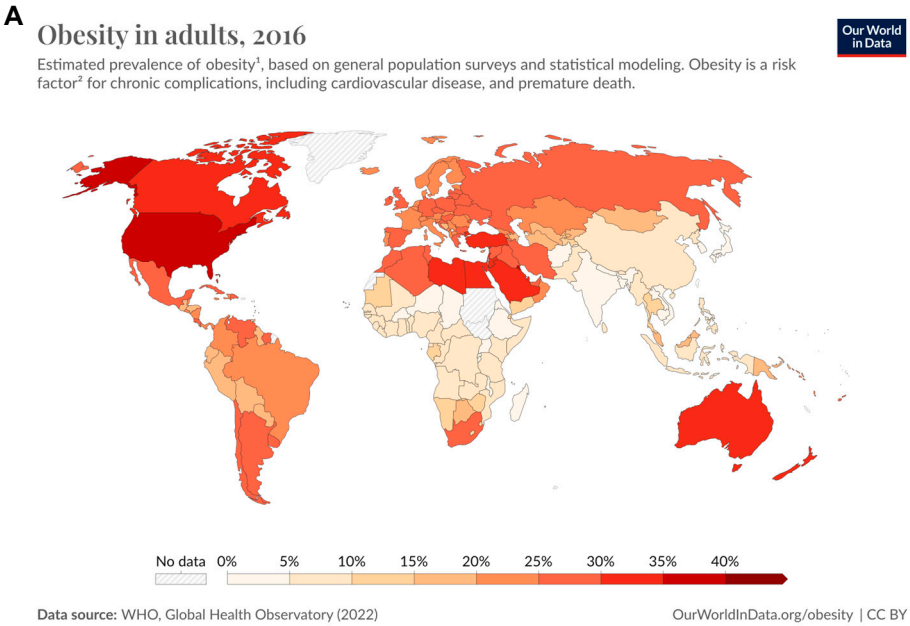
1.2.1. The obesity pandemic

Even though humanity managed to cope with the COVID-19 pandemic, there is still another pandemic around – obesity, the prevalence of which is constantly increasing [14, 15]. The prevalence of obesity has grown significantly over the past few decades and continues to increase, reaching about 20% in Europe and over 40% in the United States (**Figure 2**) [16-20].

Obesity, according to World Health Organisation, is a chronic complex disease defined by excessive adiposity (fat accumulation) that can impair health (code 5B81 in the International Classification of Disease ICD-11) [18, 21, 22]. It is assessed based on *body mass index* (BMI), calculated as described in the equation below. A condition with BMI between 25.0 and 29.9 kg/m^2 is considered as *overweight*, and obesity is diagnosed when BMI exceeds 30 kg/m^2. Besides BMI, additional measurements, such as waist circumference, are often used for diagnosis determination [18].

$$BMI = \frac{weight\ (kg)}{height^2\ (m^2)}$$

The latest assessment of various risk factors negatively affecting human health by the Global Burden of Diseases study found that high fasting plasma glucose and high body mass index are the third and fourth most dangerous risk factors, attributing to 6.53 and 4.72 million deaths globally per year, respectively. Meanwhile, high systolic blood pressure and smoking occupy the first two positions among risk factors, accounting for 10.4 and 7.10 million deaths per year, respectively. This creates a huge burden on the healthcare system and negatively



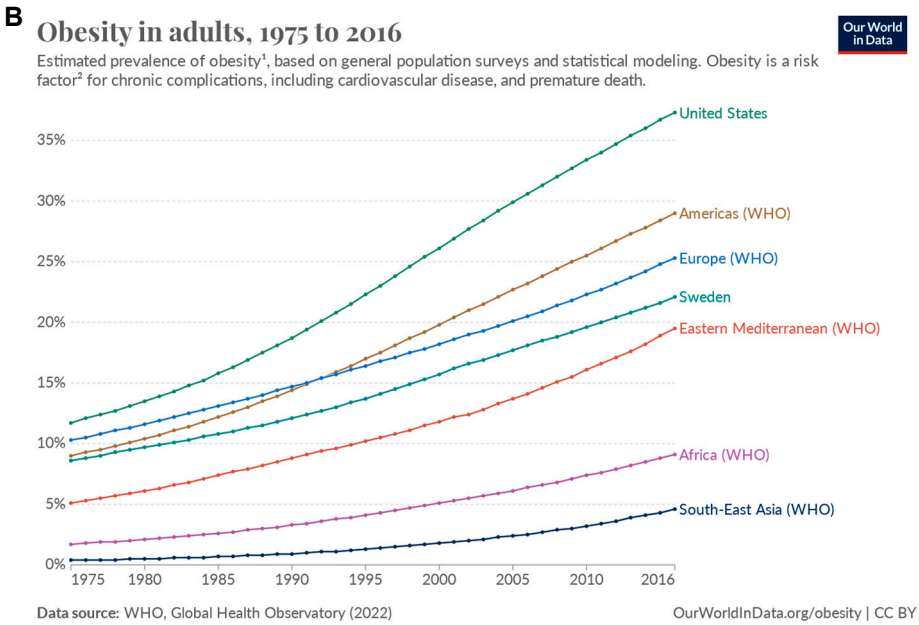


*Figure 2. The share of people with obesity (body mass index of 30 kg/m^2 or higher) has grown rapidly since the 1970s. (**A**). The estimated prevalence of obesity in adults in 2016. (**B**) The chart illustrating the changes in the prevalence of obesity from 1975 to 2016.* | Both plots are based on the data from World Health Organization - Global Health Observatory (2024) data repository - processed by Our World in Data.

affects human lives in general [23]. Additionally, it was demonstrated, that the hazard ratio for overall mortality for people with BMI in the range of 30.0 to 34.9 kg/m^2 was increased by 44%, and for BMI >40 kg/m^2 was almost twice as high as for people in the healthy BMI range of (18.5-24.9 kg/m^2) [24].

Obesity has several harmful effects on health, such as increased risks for the development of type 2 diabetes (T2D), cardiovascular diseases (CVDs), and some types of cancer [23, 25]. For example, the results of the Swedish Obese Subjects study (SOS), which followed two groups of people for up to 20 years – the group who underwent weight-loss surgery (n = 2010) and a control group under usual care (n = 2037) – demonstrated that the weight-loss group had significantly reduced overall mortality (-30%) and lowered incidences of CVDs (-30%) and diabetes (-80%) (**Figure 3**) [25]. Additionally, T2D also negatively affects human health by increasing the risk of several diseases, among which the most common are heart attack, stroke, kidney failure, retinopathy, and lower limb amputation [26]. The strong association between obesity and T2D has even lead to the introduction of term "*diabesity*" [27].

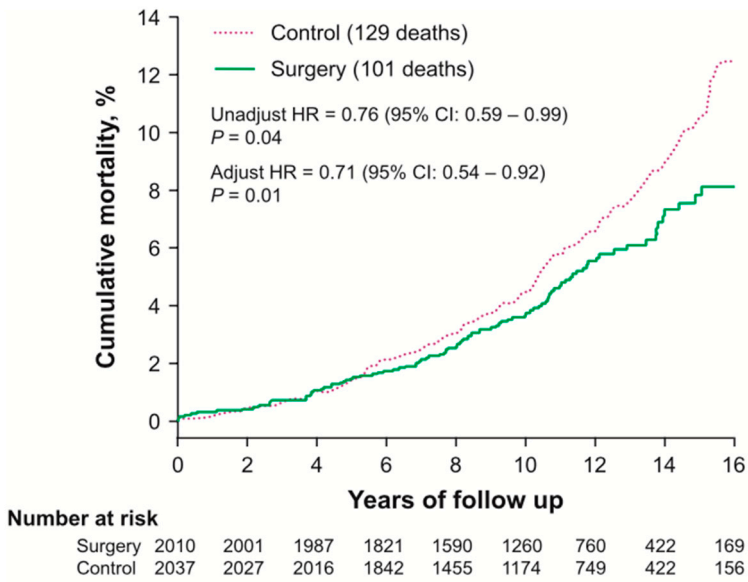


Figure 3. Cumulative mortality in two groups of participants: control group under usual care and a surgery group, in which participants underwent weight-loss surgery. | Reproduced with permission from Sjöström, L. et al. [25]. Copyright © 2012, John Wiley and Sons.

1.2.2. Metabolism in healthy individuals

In a healthy condition, when glucose gets into the bloodstream after a meal, insulin is secreted by the pancreatic β-cells to promote peripheral tissues to absorb incoming glucose [28]. The summary of the processes happening under insulin secretion is given in **Figure 4** [28]. Insulin binds to the insulin receptor in peripheral tissue, which initiates a cascade of biochemical processes, including activation of glucose transporters, facilitating glucose uptake and activation of *glycolysis* (glucose breakdown). At the same time, insulin is known as an *anabolic* hormone, which promotes energy storage. Thus, insulin facilitates the conversion of excess incoming glucose into fat (mainly consisting of triglycerides, TGs – the primary form of energy storage in the body) in adipose tissue through the process of *lipogenesis*. Insulin also stimulates glucose deposition in the form of *glycogen* (a polysaccharide made of glucose units – the storage form of glucose) in the process of *glycogenesis* in the liver and muscles, and reciprocally suppresses *glycogenolysis* (glycogen breakdown). At the same time, *gluconeogenesis* (formation of new glucose molecules) is also suppressed by insulin after a meal.

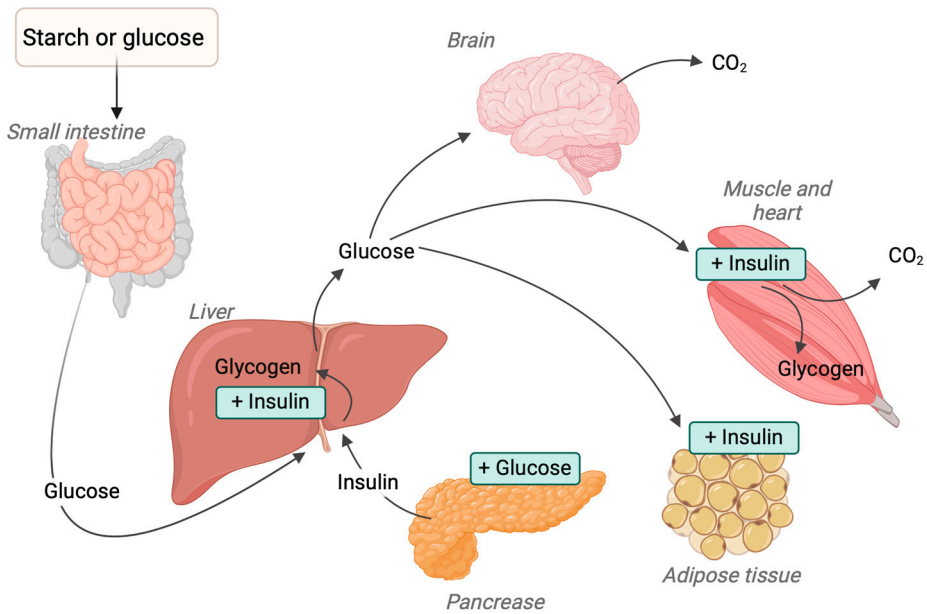


Figure 4. Overview of the key processes involved in carbohydrate metabolism after a carbohydrate-rich meal. | Redrawn with permission from Frayn, K. and Evans, R. [28]. Copyright © 2019, John Wiley and Sons.

The tendency of the body to store glucose in the form of easily accessible glycogen and to not convert all excess glucose into fat can be explained by the critical role of

glucose for some tissues and organs in the body, such as red blood cells and brain cells, which require glucose as an primary energy source and cannot utilise, for example fat [29]. The reason for this is that red blood cells do not have mitochondria, which are required for lipid oxidation, and the brain cannot utilise fatty acids (FA), because they cannot cross the blood-brain barrier (although the brain can utilise ketone bodies formed from FA). In addition, TGs can only be used to synthesis glucose to a limited degree, for example, glycerol formed during TG breakdown can be utilised for gluconeogenesis, but the amount of glycerol in stored TGs is extremely small. Considering that blood cells and the brain are crucial for body functioning and require a significant amount of energy (approximately 20% of total energy intake is consumed by the brain), glycogen represents an "emergency" fuel source for the body. An overview of the integration of carbohydrates, fats, and protein metabolism is given in **Figure 5** [28].

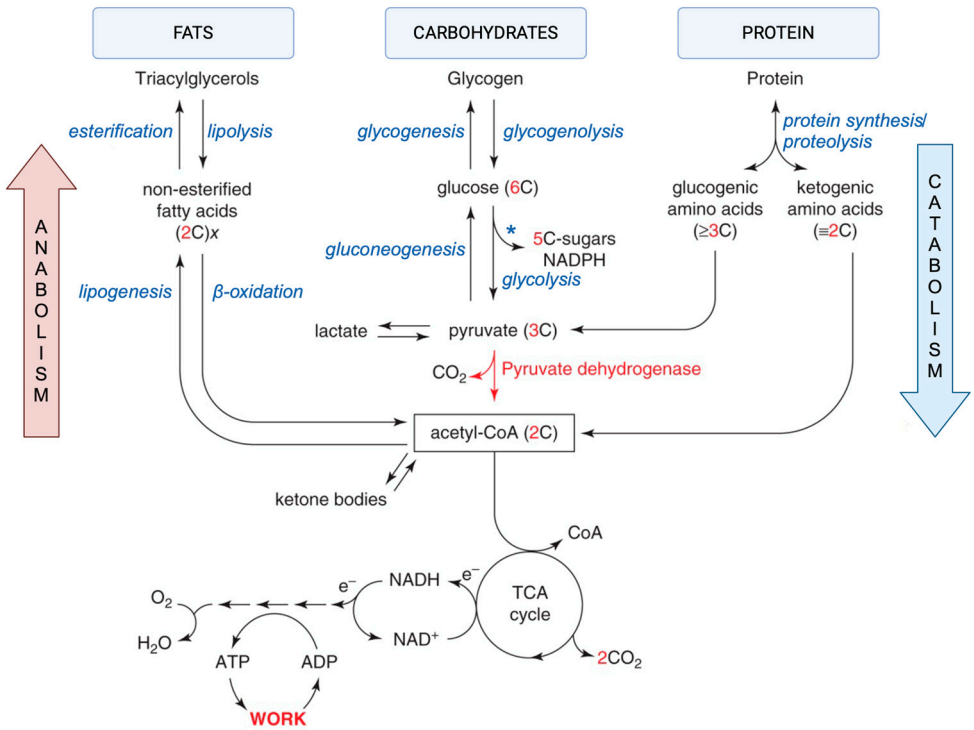


*Figure 5. Integration of carbohydrate, fat, and protein metabolism in the body. * – pentose phosphate pathway.* | Reproduced with permission from Frayn, K. and Evans, R. [28]. Copyright © 2019, John Wiley and Sons.

1.2.3. Type 2 diabetes and metabolic dysfunction-associated steatotic liver disease

Type 2 diabetes is characterised by *insulin resistance* – a state in which peripheral cells become less sensitive to insulin, which results in their inability to take up glucose from the blood (**Figure 6**) [30, 31]. Initially, the pancreatic beta-cells can compensate for the increased insulin resistance by increasing the secretion of insulin (**Figure 7**) [28, 32]. However, in some individuals, who may be genetically predisposed, over time, the beta-cells fail to do so, causing blood glucose levels to increase and T2D to develop.

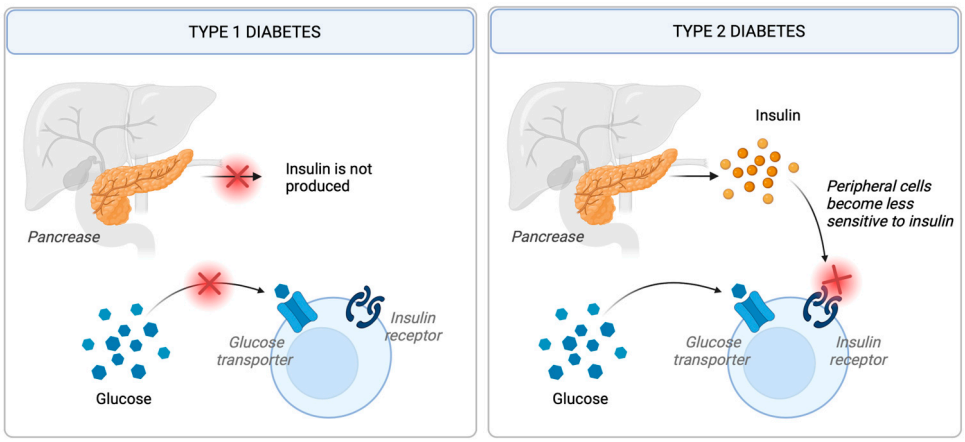


Figure 6. Key difference between type 1 and type 2 diabetes: T1D is characterised by the pancreas's almost complete inability to produce insulin, whereas in T2D, insulin is produced but peripheral cells develop insulin resistance and become less sensitive to it.

Obesity increases the risk of T2D development via increased insulin resistance, but the molecular mechanism underlying this is not fully understood, and there are several theories explaining this consequence. For instance, the negative effect of obesity has been linked to the concept of lipotoxicity [33]. Human adipocytes have a certain limit of size and amount of fat they can store. When this limit is reached, fat starts accumulating in other tissues, which do not have fat storage functions, thereby negatively affecting the function of these tissues [34]. At the same time, adipose tissue produces various pro-inflammatory cytokines such as interleukine-6 (IL-6) [35] and tumour necrosis factor-alpha (TNF-α) [36], which affect the functions of other cells and tissues, potentially leading to their dysfunction and, consequently, the development of T2D and related complications, such as CVDs, retinopathy, nephropathy, and metabolic dysfunction-associated steatotic liver disease [37, 38].

Excess fat accumulation in the liver (*steatosis*) leads to the development of *metabolic dysfunction-associated steatotic liver disease* (MASLD, previously

known as non-alcoholic fatty liver disease – NAFLD) [39, 40]. The progression of MASLD can result in inflammation (hepatitis), fibrosis, and may even lead to cirrhosis and cancer development. The mechanisms of MASLD development and progression are not yet fully understood, and no specific pharmaceutical treatment options are available [41]. Nevertheless, a combination of a healthier diet and exercise has been shown to be an effective means of combating MASLD [42].

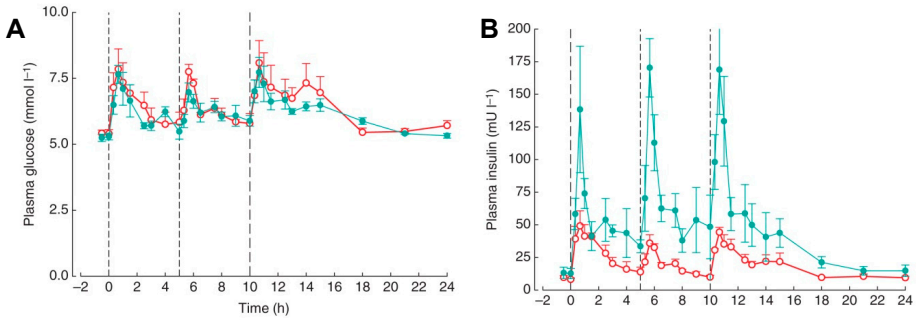


*Figure 7. Levels of plasma glucose (**A**) and insulin (**B**) in lean (red open circles) and obese individuals (filled green circles) over an ordinary day with indicated intakes of breakfast, lunch, and dinner. Although both groups of individuals have similar glucose levels, the insulin level in obese individuals is much higher, indicating an insulin resistance state.* | Reproduced with permission from Frayn, K. and Evans, R. [28]. Copyright © 2019, John Wiley and Sons. Based on McQuaid, S. E. [32]. Copyright © 2010, American Diabetes Association.

1.2.4. Type 1 diabetes

In contrast to T2D, *type 1 diabetes* (T1D) usually develops early in life and is characterised by beta-cell destruction and an often complete depletion of the pancreas's ability to produce insulin, which is crucial for modulating peripheral tissues to activate glucose transporters and allow glucose to enter cells (**Figure 6**) [30]. In untreated T1D, a *catabolic state* develops, characterised by increased breakdown of stored fuel [28]. The absence of insulin results in peripheral tissues not receiving a glucose supply, leading to stimulated glycogen breakdown and reduced glycogen formation. Additionally, the process of gluconeogenesis is stimulated, while glycolysis is reduced. To perform gluconeogenesis, the body utilises amino acids from protein breakdown (mainly from muscles) and glycerol formed during lipolysis. Thus, glucose from food cannot be utilised by tissues due to the absence of insulin, while glucose production in the body is increased. Together, these processes lead to the state of *hyperglycaemia*, characterised by increased glucose concentration (healthy baseline levels of 5 mmol/L rise to 10-20 mmol/L or more). Excess glucose in the blood can be partially excreted in the

urine – a characteristic of diabetes known as *osmotic diuresis* – which requires additional water, often leading to patients feeling excessively thirsty.

However, not only glucose but also lipid metabolism is significantly affected in T1D [28]. In the absence of energy supply from glucose, lipolysis in the adipose tissue is activated. This leads to increased blood lipid concentrations. Lipolysis produces FAs, which cannot enter into the tricarboxylic acid cycle (TCA) because the substrates required for this are rapidly consumed in gluconeogenesis. Consequently, the liver converts the FAs into *ketone bodies*: β-hydroxybutyrate, acetoacetic acid and their spontaneous breakdown product acetone. Ketone bodies can be utilised as a source of energy by most tissues in the body. However, their blood concentration in T1D patients may rise very high and lead to a decrease in blood pH; this condition is known as *diabetic ketoacidosis*. Moreover, elevated levels of glucose and ketone bodies in the blood lead to increased blood osmolarity, which, combined with the increased acidity, can affect brain and heart function and lead to unconsciousness and even *diabetic coma*.

1.3. Strategies for weight loss and improved metabolic health

Metabolic health is a loosely defined term but is generally assumed to reflect a state opposite to metabolic dysregulation, often described as metabolic syndrome and established based on the combination of several parameters, such as waist circumference, blood glucose level, blood pressure, blood cholesterol, and triglyceride levels within a healthy range. The state when at least three of these parameters are elevated is defined as the *metabolic syndrome*, which significantly increases the risk of developing T2D and CVDs [43]. The thresholds of these parameters are: waist circumference ≥102 cm for men and ≥88 cm for women, fasting plasma glucose ≥6.1 mmol/L (110 mg/dL), triglycerides ≥1,7 mmol/L (150 mg/L), reduced HDL cholesterol <1.03 mmol/L (40 mg/L) in men and <1.29 mmol/L (50 mg/L) in women, and blood pressure ≥130/≥85 mmHg.

Reducing body weight by 5-10 kg significantly reduces the risk of developing CVDs and T2D [43] and thus improves metabolic health. Different approaches can be used to improve metabolic health by reducing body weight, ranging from diets, increased physical activity, use of medications or dietary supplements, and, in a radical way, surgery [27, 44, 45]. This thesis is focused on the following approaches: the effect of the most radical surgical treatment was studied in **Paper I**; in **Paper II**, we evaluated the influence of caffeine and its metabolites on lipid accumulation in the liver – which is associated with obesity and T2D; and the dietary approach was examined in **Paper III**.

1.3.1. Why do people gain weight?

To find an effective treatment option for a disease, it is important to understand what causes it and how it develops [46]. Genetic variation is often discussed as an important factor in the development of obesity [47]. However, several studies demonstrate that genetic predisposition to some diseases can be significantly affected by lifestyle. For example, a study with data on 55 685 participants estimated the influence of lifestyle on the risks of coronary artery disease and discovered that, in the group of people with high genetic risk, a healthy lifestyle could decrease the risk of disease prevalence by 46% [48].

The development of overweight and obesity is connected to an extremely complex combination of several biological and environmental factors; some of which are shown in **Figure 8** [15]. The UK Foresight Program "Tackling Obesities" identified seven main clusters affecting obesity development in an individual or a group: physiology, individual psychology, individual physical activity, food consumption, food production, social psychology, and the physical activity environment [49]. At the same time, Matthias Blüher highlights in his review that "*Obesity is not caused by personal choice or by society but rather by the relationship between an individual and their environment*" [15].

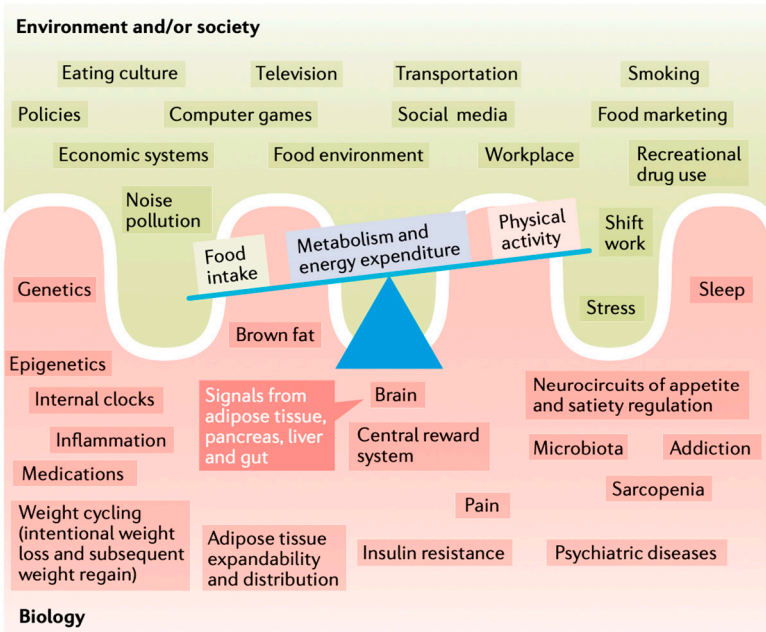


Figure 8. Environmental, societal, and biological factors contributing to obesity development. | Reproduced from Blüher, M. et al. [15] with permission from Springer Nature. Copyright © 2019, Springer Nature.

The increase in obesity rates over the last decades is usually associated with changes in society, such as higher consumption of convenience foods, an increasing share of computer-based work and entertainment activities, decreased physical activity, more persuasive food marketing and promotion of larger portions and frequent snacking, as well as normalisation of consumption of sweets, soft drinks, and fast food [15]. For example, ultra-processed food was shown to result in approximately 500 kcal per day higher food intake and led to an increase in body weight of 0.9 kg over two weeks in the study where two groups received either ultra-processed or unprocessed meals. The meals were designed to be equal in calories, sugar, fat, fibre, and other macronutrients, and participant were allowed to consume as much as they desired. (**Figure 9**) [50].

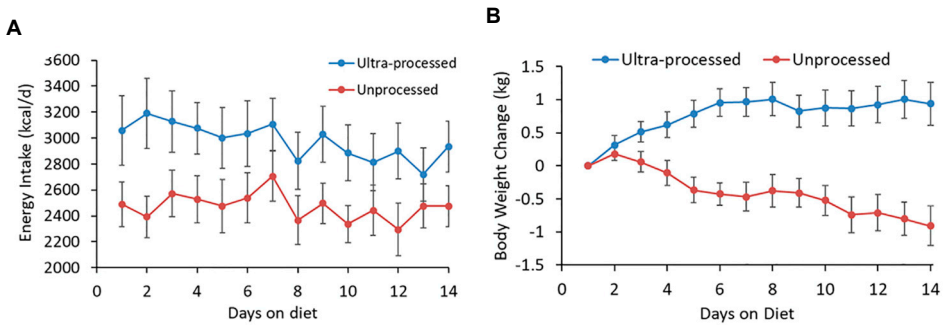


*Figure 9. The changes in (**A**) total energy intake and (**B**) body weight in groups on ad libitum ultra-processed and non-processed food diets.* | Reprinted from Hall, K.D. et al. [50] with permission from Elsevier. Copyright © 2019, Elsevier.

1.3.2. Lifestyle modification

Excess body fat accumulation occurs when the amount of caloric intake exceeds expenditure [45]. Thus, weight loss can be achieved by increasing physical activity and reducing food intake. Lifestyle modification, including weight loss (aiming at a minimum of 5%), restriction of total (<30%) and saturated (<10%) fat intake, increased intake of fibre (≥15 g/1000 kcal) and physical activity (>4 hours per week) was shown to be effective in reducing the risk of developing T2D by 58% after 4 years of follow-up in the Finnish Diabetes Prevention Study, involving 522 middle-aged overweight individuals [51]. In the US Diabetes Prevention Program [52], 3234 nondiabetic participants with elevated blood glucose and BMI >24 without diabetes were divided into three groups: a control group, a group receiving metformin and a lifestyle modification group, and followed for 4 years. The lifestyle modification included the requirement to lose 7% of body weight, follow a low-calorie, low-fat diet, and perform 150 minutes of physical activity per week. The study found that lifestyle modification was significantly more effective in reducing the risks of developing diabetes than metformin – lifestyle modification reduced the

risk by 58% and metformin by 31%. The cumulative share of people who developed T2D after 3 years in the control group was 28.9%, in metformin group 21.7%, whereas in lifestyle modification group it was 14.4% (**Figure 10**). Additionally, further evaluation of the US Diabetes Prevention Program data led to the conclusion that every kilogram of weight loss results in a 16% reduction in the risk of developing diabetes [53].

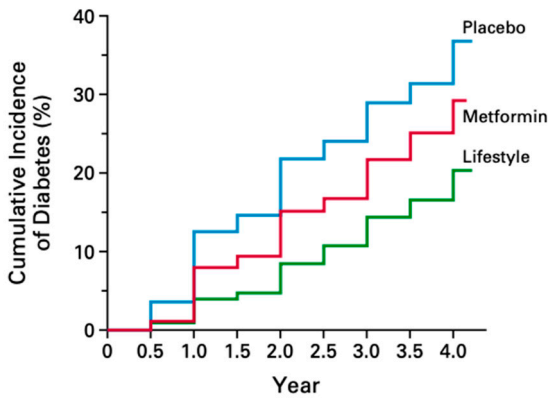


Figure 10. Cumulative incidence of diabetes in the control/placebo group, the group receiving metformin, and the group following the lifestyle modification program over four years. | Reproduced with permission from Knowler, W. C. et. al. [52]. Copyright © 2002, Massachusetts Medical Society.

Thus, lifestyle modifications to improve metabolic health typically involve weight loss. Numerous diets have been suggested for weight loss, but they can be grouped into three categories based on the used strategy, as suggested by Freire [54]: *1)* manipulation of macronutrient content, including diets focused on reducing or increasing the proportion of specific food components, e.g., low-carbohydrate or low-fat diets; *2)* manipulation of timing, including restrictions on the times when one can eat, e.g., time-restricted feeding, periodic fasting (5:2), or alternate-day fasting; *3)* restriction of specific foods, such as plant-based, Mediterranean, and Paleo diets. An overview of efficiency of different weight-loss approaches was also demonstrated in the meta-analysis of 80 weight-focused randomised controlled trials lasting for a minimum of one year by Franz et al. (**Figure 11**) [55].

A challenge when interpreting dietary studies is the imbalance between the large number of observational studies (in which subjects are observed over a period of time without the intervention of researchers) and the insufficient number of randomised controlled trials (in which participants are divided into control and experimental groups). However, even in randomised controlled trials, there is a significant challenge to control the actual food intake by participants and their adherence to the prescribed protocol [56-58]. In addition, there is a lack of long-term studies: most nutritional studies run for only a few months.

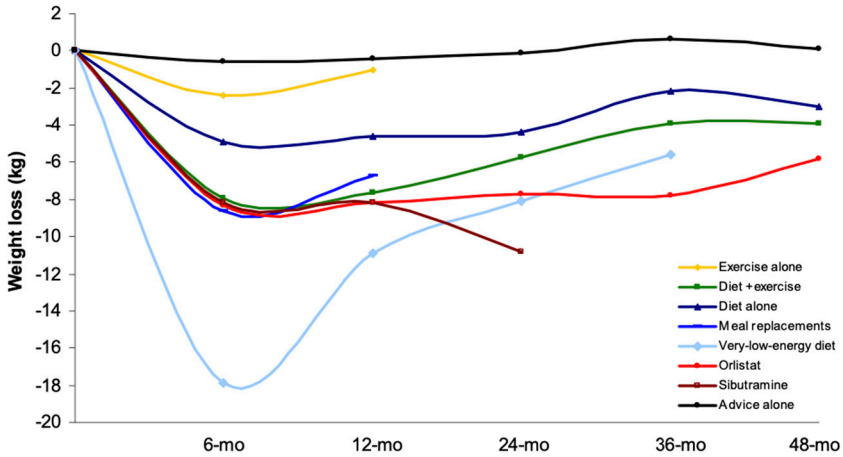


Figure 11. Average weight loss in groups of people who used different weight loss approaches for a minimum of one year. | Reprinted from Franz, M.J. et al. [55] with permission from Elsevier. Copyright © 2007, Elsevier.

Another crucial aspect of food-limiting diets, such as low-carbohydrate or low-fat diets, is the types of food used to replace the restricted macronutrients. This aspect is not always considered in diet interventions, which makes the study outcomes unclear. For example, people on a low-fat diet may increase carbohydrate consumption, and thus the observed study outcomes can be influenced by any of these modifications. To complicate matters further, the type of specific macronutrient is also important. In the case of increased carbohydrate consumption, increasing the fibre content in the diet can be beneficial for health, whereas an increase in refined and high glycaemic index carbohydrates would have a negative effect. Furthermore, food is inherently complex and is estimated to consist of more than 26000 different molecules, while only some of them (e.g., only 150 nutritional components are tracked by the United States Department of Agriculture) have been investigated in research studies [59].

To minimise these difficulties, in **Paper III** we studied the immediate effects on the human lipidome and metabolome elicited by meals rich in carbohydrates, protein, fat, and fibre in individuals with T1D and T2D and compared these effects with those in individuals without diabetes. Although the study only examined a single meal and did not involve a long-term intervention, it can be assumed that the consequences of altered dietary habits would be reflected in the cumulative effect of many individual meals. Importantly, the study utilised realistic meals, rather than examining separate food components in isolation. This approach provides a clearer picture of how the human metabolome responds to different meals and helps determine which meal types are more advantageous depending on an individual's glycaemic status.

1.3.3. Medications and supplements

It is very difficult for people to adhere to lifestyle modifications, and weight loss resulting from non-pharmacological interventions is very challenging to sustain for the majority of people [27]. For example, a meta-analysis of 45 randomised controlled trials involving 7788 individuals demonstrated that behavioural interventions focusing on modification of both food intake and physical activity resulted in a very modest average loss of 1.56 kg after 12 months [60]. In addition, there is also a lack of access to healthcare professionals providing obesity treatment and limitations associated with the cost of guided weight-loss programmes [61]. Thus, several alternative strategies have been developed. Among them, various supplements have been suggested to facilitate weight loss; however, there is a lack of sufficient evidence regarding their efficacy [61-63]. These dietary supplements include, for example, chocolate/cocoa, calcium and vitamin D, *Camellia sinensis* (green tea), *Phaseolus vulgaris* (white kidney bean), *Garcinia cambogia*, *Ephedra sinica*, caffeine, chitosan, conjugated linoleic acid, and guar gum [61, 63]. A systematic review of dietary supplements and alternative therapies used for weight loss found only 16.5% – or 52 studies out of 315 randomised controlled trails – to have a low risk of bias and to be sufficient to support the efficacy of examined treatment options [61]. Among them, only 16 studies showed significant weight changes in the range of 0.3 to 4.93 kg. Similar results were obtained by another research group in their meta-analysis of randomised controlled trials of herbal medicines and dietary supplements containing isolated organic compounds [63]. It was found that some dietary supplements and herbal extracts produced significant weight loss, although weight loss for all of these supplements was bellow 2.5 kg.

The development of anti-obesity medication began several decades ago but has been extremely challenging. Only recent progress in understanding the mechanism of appetite regulation has stimulated the development of new anti-obesity drugs [64]. Several anti-obesity drugs that reached the market were withdrawn later due to adverse reactions. Some of the medications available today can be used only for short-term treatment due to the potential development of adverse effects, such as addiction or tolerance.

Anti-obesity drugs, which are now available on the market, have different mechanisms of action [64]. For example, phentermine and cathine are sympathomimetic medications, which mimic the effects of endogenous agonists on the sympathetic nervous system. Phentermine is also used in combination with the anticonvulsant drug topiramate, due to its hunger reducing effect. Another known combination includes the opioid receptor agonist naltrexone with bupropion, a dopamine and noradrenaline reuptake inhibitor. Orlistat, which is available over the counter, reduces fat absorption from food due to its action as an intestinal lipase inhibitor.

The most novel class of anti-obesity drugs is the glucagon-like peptide-1 (GLP-1) receptor agonists, such as liraglutide and semaglutide. Before the approval of semaglutide, the available anti-obesity drugs were able to provide weight reduction only within a single-digit range. Semaglutide, approved by the FDA in 2021, marked a breakthrough in the field of anti-obesity drugs by offering significantly higher weight loss and sufficient tolerability [64]. Semaglutide was demonstrated to reduce body weight by 14.9% after 68 weeks of treatment in patients without diabetes [65] and by 9.6% in patients with T2D [66]. However, semaglutide is prescribed for the treatment of overweight or obesity when combined with at least one comorbidity, such as T2D, high blood pressure, or high cholesterol levels [64].

The mechanism of action of semaglutide is based on its action as an agonist of GLP-1, which belongs to the group of gastrointestinal tract hormones – the *incretins* [67]. Incretins are released by the intestine after food intake and promote insulin secretion by the pancreas and also reduce gastric emptying and food intake. Semaglutide has a complex effect on several systems in the body. It reduces gastrointestinal motility and gastric emptying, affects the brain by reducing reward behaviour and food intake; it stimulates insulin secretion from the pancreas and enhances insulin receptor signalling and glucose uptake by muscles, altogether providing appetite reduction and improvements in insulin sensitivity and metabolic health [67, 68].

Several other drugs (over 30, according to a 2022 review by Müller et al.) are now in the development stage for the treatment of obesity [64]. Most of them have mechanisms of action related to gastrointestinal hormones.

The majority of anti-obesity drugs require a prescription and are quite expensive, reducing their availability to many people. Additionally, anti-obesity drug intake can be associated with side effects. Thus, food supplements remain a popular alternative for weight management and improvement of metabolic health. Caffeine and coffee are among these food supplements suggested to improve human health, facilitate the reduction of blood glucose levels, and have beneficial effects on liver health [69]. In **Paper II**, we studied the influence of caffeine and its metabolites on fatty liver disease, which is associated with obesity and T2D.

1.3.4. Weight-loss surgeries

In a more radical approach, obesity can be treated with surgery, which can be performed in different ways. Several weight-loss (or bariatric) surgical procedures have been developed to reduce the size of the stomach, limiting the amount of food people can eat (**Figure 12**) [70]. For example, in sleeve gastrectomy, part of the stomach is removed, in gastric banding, an adjustable band is placed over the stomach, reducing its size, and in *gastric bypass*, or *Roux-en-Y gastric bypass surgery* (RYGB), the stomach size is reduced to about 30 ml and the upper part of

the small intestine is bypassed, thus reducing not only the amount of food one can eat but also its absorption [71].

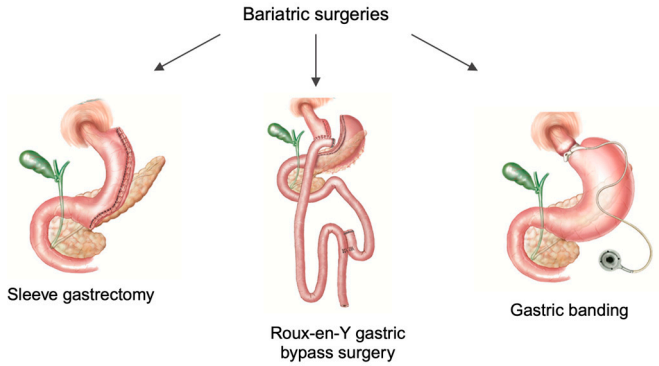


Figure 12. Examples of the most common bariatric (or weight-loss) surgeries. | Reprinted from Nuzzo, A. et al. [70] with permission from Elsevier. Copyright © 2020, Elsevier.

RYGB is one of the most common bariatric surgeries [71] and provides the most significant reduction in body weight during the first year after the surgery (**Figure 13**) [72]. Moreover, bariatric surgeries were shown to reduce overall mortality by 29%, the risk of myocardial infarction by 29%, the risk of stroke by 34%, the risk of cancer in women by 42% and the risk of developing T2D by 83% [25]. Furthermore, in 87% of cases, it also results in improvements in blood glucose levels in people diagnosed with T2D at the time of surgery [73]. The metabolic effects of RYGB were studied in **Paper I**.

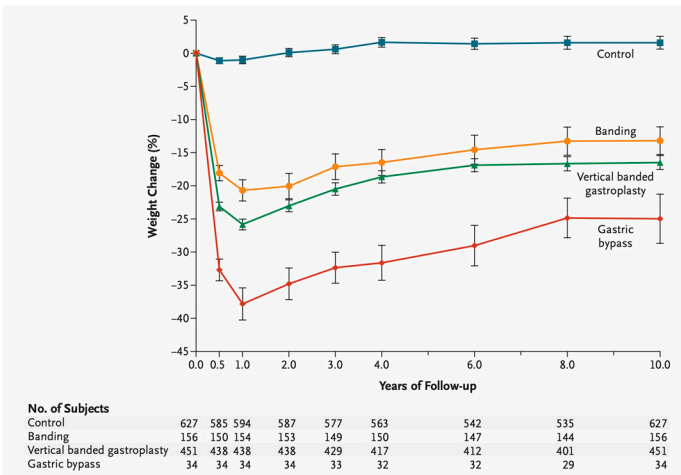


Figure 13. Weight change (%) in response to different bariatric surgical procedures. | Reproduced with permission from Sjöström, L. et al. [72]. Copyright © 2002, Massachusetts Medical Society.

1.4. Application of metabolomics and lipidomics to study diabetes and obesity

A better understanding of the mechanisms involved in diabetes development and the negative consequences of obesity on health is necessary to develop effective treatment strategies, including new drugs and guidelines for lifestyle modification. In this process, metabolomics and lipidomics play an important role, as these approaches allow for monitoring of changes in numerous biologically active molecules in the body occurring under the influence of endogenous (e.g., genetic) and exogenous (e.g., lifestyle and diet) stimuli [74]. Identified alterations can, besides offering knowledge on the mechanisms of disease, become biomarkers for disease diagnosis.

Metabolomic studies provide detailed descriptions of a disease far beyond the single biomarkers commonly used in clinical practice, thereby leading to a better understanding of a disease. Clarification of the underlying biological mechanisms of a pathological process can in turn reveal novel targets for drug development [75]. For example, diabetes impacts the metabolism of all macronutrients, but it is defined only by glucose levels. However, glucose alone is incapable of providing a full description of the underlying metabolic dysregulation. Thus, metabolomics and lipidomics, focusing on the analysis of numerous compounds, are efficient tools for understanding diabetes and related conditions. For example, metabolomics was used to distinguish maturity-onset diabetes of the young type 2 (MODY2) from other types of MODY and T2D and provided a deeper understanding of MODY2 [76].

Metabolomics and lipidomics are considered to be more cost-effective and productive in the process of drug discovery and development compared to a process starting from finding a dysregulated gene (then a protein and then a potential drug that can regulate this shift). The reason for this is that not all diseases have a genetic basis and, in many cases, environmental factors are crucial for disease development [75]. This is supported by the example of the recent discovery of the role of trimethylamine-N-oxide (TMAO) in the development of atherosclerosis. [77]. This discovery was followed by the identification of enzymes involved in the formation of TMAO and led to the rapid development of the corresponding inhibitor [77]. Moreover, dysregulation of metabolism is also a hallmark of cancer [78], and is likely to be involved also in many other diseases, such as Alzheimer's disease, autism, schizophrenia, and inflammatory bowel disease, all of which are being increasingly studied using metabolomics [75].

In the field of diabetes research, metabolomics studies led to the discovery that branched amino acids (leucine, isoleucine, and valine), as well as aromatic amino acids (phenylalanine and tryptophan) and 2-aminoadipic acid, are elevated in individuals with T2D and can be used to identify individuals with a high risk of developing T2D up to 12 years before the disease onset [79, 80].

In the lipidomics field, insulin resistance has been linked to alterations in ceramide levels, and their accumulation has been shown to cause changes in tissue metabolism and stimulation of apoptosis [81, 82]. Thus, the corresponding enzymes involved in ceramide production have become potential targets for the development of drugs to treat insulin resistance and other obesity-associated metabolic diseases [83]. Moreover, the measurement of certain ceramide ratios in human blood has been proven to indicate the risks of developing CVDs and has been implemented into clinical practice in Finland and at the Mayo Clinic in the US [84]. These ceramide measurements are performed on a robotic-assisted MS platform, and the cost and speed of the analysis are comparable to those of an antibody-based assay [84]. At the same time, it should be noted that the development of such ceramide tests has only become possible in recent years due to the development of advanced mass-spectrometers, which allow the measurement of ceramides present at very low concentration in blood – at the level of approximately 1/1000 of cholesterol level [83]. Thus, the constant development of analytical instrumentation introduces new opportunities in revealing novel alterations in metabolite levels associated with diseases and paves the way for new biomarkers and drugs.

The rapid development of metabolomics has also made it an important component of precision medicine [11, 85]. Metabolomics has been applied to distinguish individual responses to different dietary interventions and to establish so-called *metabotypes* – groups of people with unique responses to specific diets [86, 87], [88]. This approach allows prediction of how a particular meal will affect postprandial blood glucose in each individual, thereby helping to identify the most suitable diet for each person [89].

Thus, the rapid advancement of metabolomics instrumentation and its growing accessibility promotes the use of metabolomics in clinical practice. Even though it currently appears quite complicated for routine clinical analysis, as David S. Wishart mentioned, similar hesitations existed when introducing currently well-established analytical techniques and clinical chemistry in the middle of the 20th century [75].

2. Aims of the Thesis

The aim of the PhD thesis is to study changes in the metabolome in relation to diabetes, obesity, and MASLD, using metabolomics and lipidomics approaches to gain a better understand of strategies that can be used to treat and prevent these conditions. The thesis sub-projects were designed to evaluate interventions ranging from invasive to non-invasive, including the study of RYGB, dietary supplements, such as caffeine, and macronutrients in food, which can be used to improve metabolic health.

Specific aims:

Paper I: Currently, it is unknown how long improvements in metabolism last after RYGB. The aim of this project was to investigate metabolic remission after RYGB, including significant weight regain and normalisation of blood metabolite levels. The ultimate aim, which was not reached, was to enable the identification of individuals who will show long-term benefits from RYGB.

Paper II: Caffeine has been suggested to improve health, but both causal evidence and mechanistic understanding are lacking. The aim of this project was to evaluate the impact of caffeine on the healthy liver and potentially provide a mechanism for the suggested role of caffeine in protecting against MASLD.

Paper III: There is still debate regarding the optimal and healthiest diet, especially for people with diabetes. The aim of this project was to evaluate the alterations in the human metabolome and lipidome in response to an acute meal tolerance test, using meals enriched with either carbohydrates, fats, protein, or fibre in individuals with type 1 and type 2 diabetes, and to compare these responses to normoglycaemic individuals, in order to evaluate which dietary strategies are better for metabolic health within each group.

3. Methods

3.1. Sample preparation

Numerous procedures have been developed for extraction of metabolites and lipids from various biological samples. Some procedures suggest simultaneous extraction of metabolites and lipids into a single-phase [90, 91] or partitioning them in a two-phase system [92-94], while others are developed to extract polar and lipophilic analytes separately [95, 96]. Due to the high complexity of the metabolome and lipidome, it is usually advantageous to perform separate extractions of polar and lipophilic compounds [12]. One of the main advantages of this approach is that it helps to reduce ionisation suppression, a much-feared problem occurring with MS detection, which complicates metabolite/lipid identification and quantification [2]. For instance, in this thesis, a sample preparation based on the addition of a methanol-water mixture to blood samples [96] has been widely applied since it allows separation of targeted polar analytes from proteins and some of the lipid classes, especially glycerolipids and sterols, which could compromise the quality of the MS measurements due to extensive matrix effects and ionisation suppression (**Figure 14**). Protein removal also helps to minimise contamination of the analytical instrumentation and extend column lifetime.

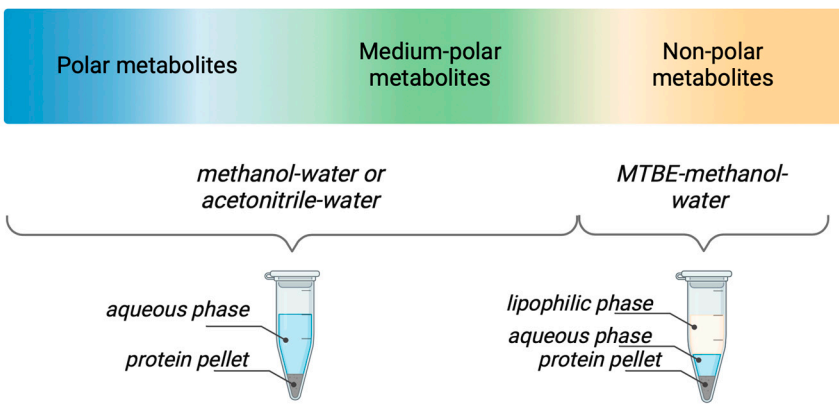


Figure 14. Sample preparation strategy applied in this thesis to obtain samples enriched with polar and lipophilic compounds. MTBE – methyl tert-butyl ether.

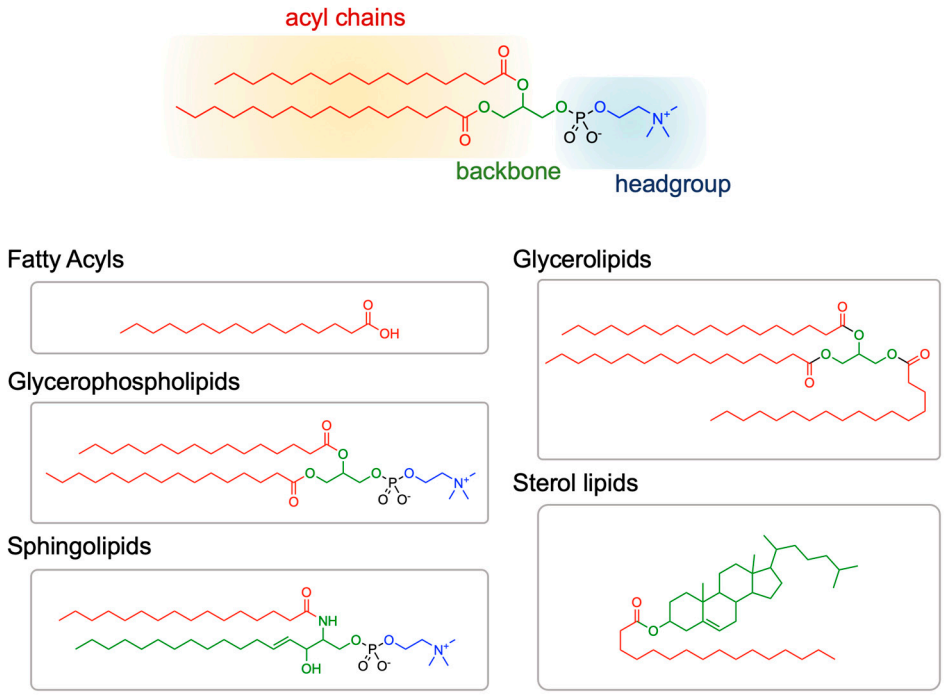


Figure 15. Examples of lipids from the most common lipid classes in humans.

There are also several strategies for the preparation of lipid-enriched samples. Lipids are divided into eight major classes (based on the structure of the backbone and headgroup), and the structures of the most common in humans are shown in **Figure 15** [97]. The solubility in various solvents differ significantly between these classes, ranging from relatively polar phospholipids to highly lipophilic triglycerides. The solubility also varies within each class and depends on the number of acyl chains and the number of carbons in these chains.

Extraction of lipids can be performed using a single-phase extraction approach [95]. However, this approach has been shown to extract mainly polar lipids, whereas a significant amount of non-polar lipids (such as triglycerides and cholesterol esters) can be lost [98]. There are also several two-phase extraction procedures, e.g., traditional chloroform-based methods [99, 100] or methods based on methyl *tert*-butyl ether (MTBE) [101], which are the most common nowadays. A modified version of the MTBE-based protocol developed by Matyash et al. [101] was used in this thesis [102]. Two-phase extractions require slightly more preparation time but allow for the extraction of more lipid classes and the reduction of sample complexity by the removal of proteins, salts, and most polar metabolites [98].

3.2. Chromatographic separation

Extracted samples can be analysed directly by MS or by a combination of chromatography with MS. The high complexity of the metabolome and lipidome, along with the presence of numerous isomeric compounds, existing in a relatively narrow mass range of about 200-1200 Da, makes it advantageous to use chromatographic separation prior to MS [2, 12].

Chromatography is a technique that allows the separation of analytes based on their differential distribution between a stationary and a mobile phase. The *stationary phase* is usually contained in a column through which the *mobile phase* is flowing by the aid of a high-pressure pump [103]. When a mixture of analytes is loaded onto the column, analytes with a higher distribution to the mobile phase will pass the column faster than those being mainly distributed to the stationary phase, resulting in their separation. Based on the type of mobile phase, chromatography is classified into *gas* and *liquid chromatography*, utilising gases (e.g. helium or nitrogen) or liquids (e.g. various organic solvents), respectively [103]. In liquid chromatography, separations can be performed with a non-polar stationary phase and a polar mobile phase (reversed-phase liquid chromatography, RP-LC) or with a polar stationary phase and a non-polar mobile phase (hydrophilic interaction liquid chromatography, HILIC).

The wide diversity of physicochemical properties of the metabolites and lipids often results in the necessity to use different chromatographic approaches to ensure a descent coverage of the metabolome and lipidome, respectively [10, 11]. The majority of polar small molecules present in biological samples, such as amino acids and carboxylic acids, are too polar to be retained by RP-LC and are therefore often separated by HILIC, or, following derivatisation to produce volatile derivatives of the metabolites, by gas chromatography (GC) (**Figure 16**) [12, 104, 105].

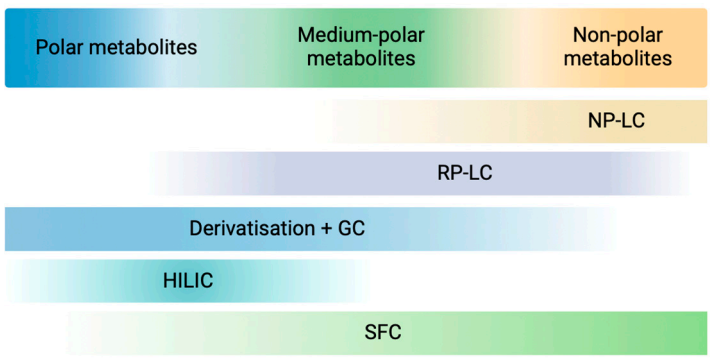


Figure 16. Different chromatographic techniques can be applied to cover wide polarity range of complex samples. | Redrawn with adaptation from Roca, M. et al. [12] with permission from Elsevier.

Chromatographic separation of lipids is usually based either on the head-group polarity or on acyl chain hydrophobicity [106-108]. *Head-group-based separation* is usually performed via normal-phase liquid chromatography (NP-LC), HILIC, or supercritical fluid chromatography (SFC), and is based on the different nature of lipid headgroups ranging from zwitterionic and polar headgroups, such as in phospholipids, to neutral and quite non-polar headgroups, such as in glycerolipids (**Figure 15**). On the other hand, *separation based on acyl chain* composition is usually achieved by RP-LC and is based on the number of acyl chains, the number of carbons in the acyl chains, and the degree of unsaturation. Lipids containing longer and more saturated acyls are retained the most in RP-LC. The fact that lipids within a particular lipid class represent a homologous series can be used to link retention times to acyl composition, thereby facilitating lipid identification [109, 110]. For example, this approach was used to increase the confidence in lipid identification in **Paper I** (**Figure 17**) and to construct the list of targeted lipids in **Paper II**.

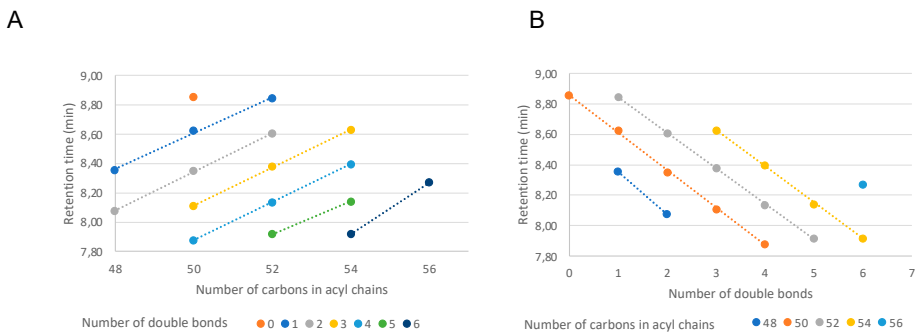


*Figure 17. Correlation of triglyceride retention time with (**A**) their acyl chains lengths and (**B**) degree of unsaturation (based on the data from Paper I).*

3.3. Mass spectrometry

MS, according to IUPAC, is the study of matter through the formation of gas-phase ions that are detected and characterised by their mass and charge [111]. The MS process involves three main steps: analyte ionisation, ion separation based on the mass-to-charge (*m/z*) ratio, and detection.

A mass spectrometer can separate only ions; thus, analytes need to be ionised prior to separation in the mass analyser. Different types of ionisation sources are used in the metabolomics/lipidomics field, such as electrospray ionisation (ESI), atmospheric-pressure chemical ionisation (APCI), atmospheric-pressure

photoionization (APPI), and electron ionisation (EI). The most common type of ionisation used in LC-MS-based metabolomics/lipidomics is ESI, and in GC-MS-based metabolomics it is EI [11, 112].

Several different mass analysers are applied in the metabolomics and lipidomics fields, such as single quadrupole (Q), time-of-flight (TOF), ion trap (IT), Orbitrap, and Fourier transform ion cyclotron resonance (FT ICR); these analysers are often combined to provide tandem MS capability [2]. Mass analysers can be grouped into low- and high-resolution types based on their ability to distinguish ions of similar mass. TOF, Orbitrap, and FT ICR mass spectrometers are often considered *high-resolution instruments* and provide high accuracy in mass measurements, whereas quadrupoles and ion trap-based mass spectrometers are considered *low-resolution instruments* [103, 112].

Lipids and metabolites can be analysed in targeted or untargeted modes [113]. *Targeted* analysis is often performed on low-resolution triple quadrupole (QQQ) instruments, which provide high selectivity and low detection limits due to the ability of the instrument to perform *selective reaction monitoring* (SRM), often referred as *multiple reaction monitoring* (MRM) in the case of several reactions [114]. This approach requires providing the instrument with a list of certain *transitions* (reactions) for each compound of interest, defining *m/z* value of a precursor ion, fragmentation energy, and the *m/z* value(s) of formed product ion(s) for each analyte of interest [115]. The principle of operation of the QQQ instrument is based on the different functions of each quadrupole: the first quadrupole is used to select the intact precursor ion, which is then fragmented in the second quadrupole (also called the "collision cell"), followed by selection of product ions in the third quadrupole (**Figure 18**). In the thesis, targeted analysis was performed on a QQQ instrument in **Paper II** for the analysis of lipids in hepatocytes due to the low amount of sample material and expected low lipid concentrations. The examples of used transitions can be found in **Table 1** and **Figures 23-24.** The limitation of such targeted methods is that they provide information only for compounds for which transitions have been predefined.

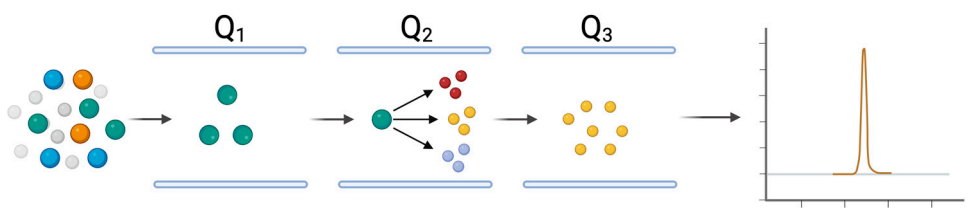


Figure 18. Principle of targeted mass spectrometry technique performed on a triple quadrupole QQQ instrument. The first quadrupole filters out a product ion, which is fragmented in the second quadruple, followed by a second filtration step were only the pre-selected product ions are allowed to pass through the third quadruple.

In *untargeted methods*, all *m/z* values within a certain range are measured, and this type of analysis is usually performed on high-resolution instruments. High-resolution instruments have a significant advantage, as they allow separation of metabolites/lipids with very close *m/z* values [107, 116].

Identification confidence in untargeted analysis can be increased by the application of *tandem MS* (or *MS/MS analysis*) [117]. In this case, two mass analysers are combined (e.g., single Q and TOF), with a collision cell in-between. Such a system allows ion fragmentation: the first mass analyser serves as a mass filter for a particular ion, which is then fragmented in the collision cell, and formed fragments are analysed in the second mass analyser. Modern instruments can perform ion fragmentation automatically in *data-dependent acquisition* (DDA) or *data-independent acquisition* (DIA) modes [118]. In DDA, after one full scan, the instrument selects the ions associated with the most intense signals and then fragment and analyse formed ions. The disadvantage of this approach is that the instrument chooses only the most intense peaks for further fragmentation, and this may result in the absence of information about less abundant species. Data-independent acquisition can be performed via e.g. all-ion fragmentation (AIF), where the instrument performs a full scan followed by a single fragmentation scan for all ionisable analytes present at that occasion. The disadvantage of DIA modes is the very high complexity of generated data and the loss of connection between precursor ions and their fragments [118].

3.4. MS coupled to ion mobility spectrometry

Additional improvements in metabolite/lipid identification can be achieved by combining an LC-MS system with *ion mobility spectrometry* (IMS) [119]. IMS provides an additional dimension for ion separation based on their *collisional cross-section* (CCS) values, which highly depend on the size and shape of the analyte, thereby providing an exceptional tool for separating isomeric compounds [120]. This approach was applied for lipid analysis in **Paper III** using *trapped IMS* (tims), which in combination with a QTOF provides detailed fragmentation data via the *parallel accumulation serial fragmentation* (PASEF) workflow [121].

The principle of IMS is based on the separation of ions in a drift tube through which a stream of gas flows. Analytes are rendered resistance to the gas flow by the application of an electric field. The separation depends on an ion's CCS value, which describes how often a molecule collides with other molecules around it and, consequently, how fast an ion can drift in an IMS instrument. In this way, CCS values can be compared to a ship's sail: a ship with larger sails will move faster than one with smaller sails.

Although the concept of IMS was developed in the 1890s, mass spectrometers integrated with IMS were not widely applied until recently. This was due to the bulkiness of most IMS instruments, their susceptibility to ion losses, and their low duty cycles [122, 123]. A significant increase in the use of IMS-MS began in the 2000s, following the introduction of a commercial IMS-MS by Waters in 2006 [122]. Other vendors followed and recently Bruker introduced the timsTOF, which utilises an IM analyser with the PASEF workflow, followed by a high-resolution TOF mass analyser.

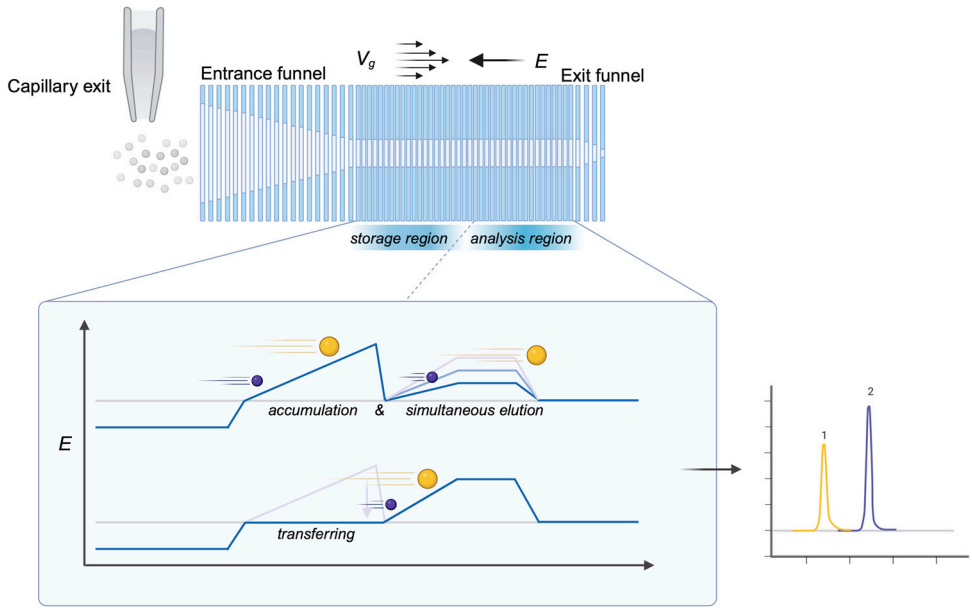


Figure 19. The principle of parallel accumulation serial fragmentation (PASEF) technique. Initially, the electric field in the "storage" region is reduced, allowing ions to accumulate in the "analysis" region. Then, electric field in the "storage" region is gradually decreased, leading to ion release and separation based on their CCS values. Simultaneously, the electric field in the "storage" region is increased, allowing ion accumulation. | Redrawn from Silveira, J.A. et al. [124] with permission from Elsevier. Copyright © 2016, Elsevier.

The key feature and advantage of the PASEF technology is the simultaneous separation of one group of ions while another group is being accumulated. This is achieved because the central part of the drift tube contains two regions: the *"storage" region* at the ion entrance and the *"analysis" region* at the ion elution side (**Figure 19**) [123]. The PASEF cycle consists of two main steps: first, the electric field in the "storage" region is reduced, allowing ions to accumulate in the "analysis" region. In the next step, the electric field in the "storage" region is increased, preventing new ions from entering the "analysis" region. Simultaneously, ions previously accumulated in the "analysis" region are released and separated

based on their CCS values, using a stepwise decreasing electric field. In this process, ions with larger CCS values and higher m/z values are released first, while smaller ions elute last. The resulting spectrum is called a *mobilogram*. The ions leaving the drift tube are then fragmented in the collision cell and detected by a high-resolution TOF mass analyser. The analysis occurs in cycles, with each cycle (e.g. 0.3 seconds for lipidomics analysis) typically consisting of one full-scan followed by several PASEF cycles (e.g., two 100 ms cycles in lipidomics analysis), including multiple MS/MS analyses in DDA mode [120].

3.5. Study design

The study design is extremely important in all types of studies, and especially in large-scale omics studies [125]. In the thesis projects, a *constrained randomization* approach [126] was used to minimise the variation in data associated with the so-called *batch effect*. Metabolomics studies are often performed on a large number of samples, which cannot be prepared and analysed simultaneously; thus, they are often separated into smaller groups – batches. Separation of samples into batches leads to increased variability in the data due to variations in instrument performance over time, possible sample ageing, etc. The constrained randomisation approach is based on the principle of grouping the main effect in a random order, while the order of other variables is not crucial [126]. For example, in **Paper I**, we analysed how the human metabolome changes in response to gastric bypass surgery over time. The study involved 148 participants, whose blood samples were taken at baseline and then after two, 12, and 60 months post-surgery. The research question was to elucidate how the human plasma metabolome changes in an individual. Thus, during analysis we kept all samples from one individual together but analysed them in a random order. Care was also taken to randomly analyse individuals with an without diabetes, which was another important outcome variable. At the same time, the specific order of individuals was not crucial (**Figure 20**).

Another important aspect of metabolomics experiments is the application of *quality control* (QC) samples, *internal standards* (IS), and regular verification of instrument performance using *system suitability tests* (SST) [127]. It is important that the QC sample represent the "average" sample being analysed. In the case of human plasma, a standard plasma sample (commercially available) can be utilised. However, standard samples are not always available, and in such case, a QC sample can be prepared by mixing fixed aliquots from each sample. A QC sample is then analysed regularly (e.g., after every tenth sample) in each analytical batch. QC samples and IS added to the samples allows for monitoring of system performance by checking mass accuracy, stability of retention time, and signal intensity etc. This information can then be used for signal normalisation if needed.

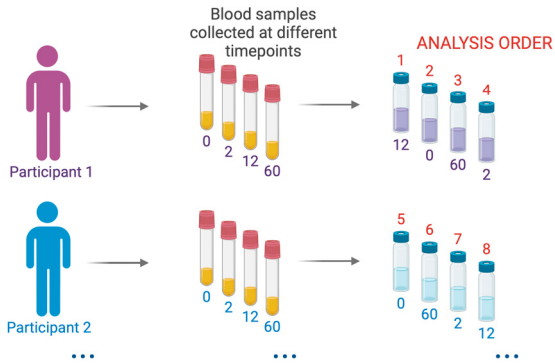


Figure 20. Illustration of constrained randomisation approach applied during sample analysis based on the Paper I study, where blood samples were collected from individuals at baseline (0) and then at 2, 12, and 60 month post-surgery.

3.6. Raw data processing

Data analysis in metabolomics/lipidomics studies consists of several steps [128] and starts with the analysis of raw data, including peak picking, which distinguishes real signals from noise, peak alignment, peak integration, metabolite identification, and sometimes quantification [129]. Compound identification is one of the biggest challenges in the metabolomics/lipidomics field due to the high complexity of these omes, the presence of isomeric compounds, coelution of analytes, and in-source fragmentation [2]. Another significant complication is the high structural heterogeneity of metabolites, which belong to multiple structural groups, making it difficult to develop systematic identification algorithms. Lipids usually have a higher level of structural resemblance, as they often can be considered as homologous series within lipid classes, but the high rate of isobaric and isomeric structures among lipids significantly complicates their identification.

Identification confidence can be improved in several different ways. First, sample complexity can be reduced by optimizing the extraction for the metabolites of interest. Additionally, orthogonal analytical data can be employed, such as chromatographic retention time, CCS-values, and mass spectra (as used for lipidomics analysis in **Paper III**). Various approaches in tandem MS can also provide additional structural information to reinforce identification of the metabolite or lipid. Furthermore, the confidence in metabolite/lipid identification should be reported according to community-established rules [13, 130, 131] and metabolite naming, especially lipid annotation, should be based on the achieved level of identification [97].

3.7. Statistical data analysis

There are numerous methods for performing statistical data analysis in the omics fields [128]. Most often the data need to be prepared for the statistical analysis and this includes operations such as data filtering, outlier detection, and transformation [132, 133]. Data filtering involves removing metabolites found in only a few samples, those with low signal levels, or those exhibiting very high variation in the QC samples. In all the studies presented in this thesis, we used log-transformed data. This transformation helps compensate for non-symmetrical data distributions around the mean, which is common in biological data, where most values are clustered around a small number but with some larger values. Log-transformation also brings data to a similar scale, thereby stabilising the variance among data (reducing heteroscedasticity) [132], as different metabolites and lipids can vary significantly in concentration in biological samples.

To evaluate how metabolites and lipids changed in response to different variables in the performed studies, we utilised different types of *linear regression models*. In **Paper II**, we used a *simple linear regression model* (SLR) to assess the effect of caffeine supplementation *in vivo*. In **Papers I** and **III** SLR was not applicable because our data were based on repeated measurements from the same individuals. Thus, we applied *linear mixed-effects models* (LMM). This type of model allows us to separate *fixed effects*, which are of primary interested (e.g., how the metabolite abundance changes over time) from *random effects*, which are less of a concern (such as differences between individuals). Additionally, LMM can be extended to multivariate LMM, where several factors are included in the model, such as time, age, sex, BMI, etc. This approach enabled us to evaluate the influence of specific factors while accounting for individual variance, determine the statistical significance of each factor. The results of these analyses can be pictured in several different ways, out of which volcano plots (e.g., **Paper II**, **Figure 2**), bar plots, and heatmaps (e.g., **Paper I**, **Figure 3**) are very illustrative.

Working with metabolomics data involves evaluating multiple interconnected metabolites and lipids. Therefore, applying *multivariate tools* that allow for an overview of all data collectively is advantageous. Multivariate analysis tools include *unsupervised* methods, such as principal component analysis (PCA), and *supervised* methods, such as partial least squares regression (PLS), also known as projections to latent structures, and orthogonal projections to latent structures (OPLS) [133-135]. The key difference between unsupervised and supervised analysis lies in the input data: unsupervised methods uses only metabolite variables without predefined grouping variables, whereas supervised methods are based on defined groups and allows for the identification of correlations between metabolites and various outcomes while removing variation in the data that is not of interest [128]. PCA is often used at the beginning of data analysis as an exploratory tool to detect any grouping in the data and identify the main sources of variation. For example, PCA

was used in **Paper I** to visualise clustering based on the time after gastric bypass surgery (**Paper I**, **Figure 2**). Meanwhile, a variant of the OPLS method based on effect projections (OPLS-EP) [126] was employed in **Paper I** to analyse the influence of acyl chain carbon number and degree of unsaturation on lipid profiles after RYGB (**Paper I**, **Figure 4**).

4. Results and Discussions

4.1. Effect of gastric bypass surgery on metabolic health – Paper I

A previous study performed in our group focused on the short-term effects of RYGB [102]. In that study, it was shown that the majority of metabolic alterations observed after RYGB result from the very-low-calorie diet that patients need to follow before the surgery. In that study, indications of the metabolome returning to initial conditions shortly after RYGB were also observed [102]. Thus, in **Paper I**, we investigated the more long-term effects of RYGB by analysing blood samples collected before RYGB and then after two, twelve, and sixty months post-RYGB (**Figure 21**).

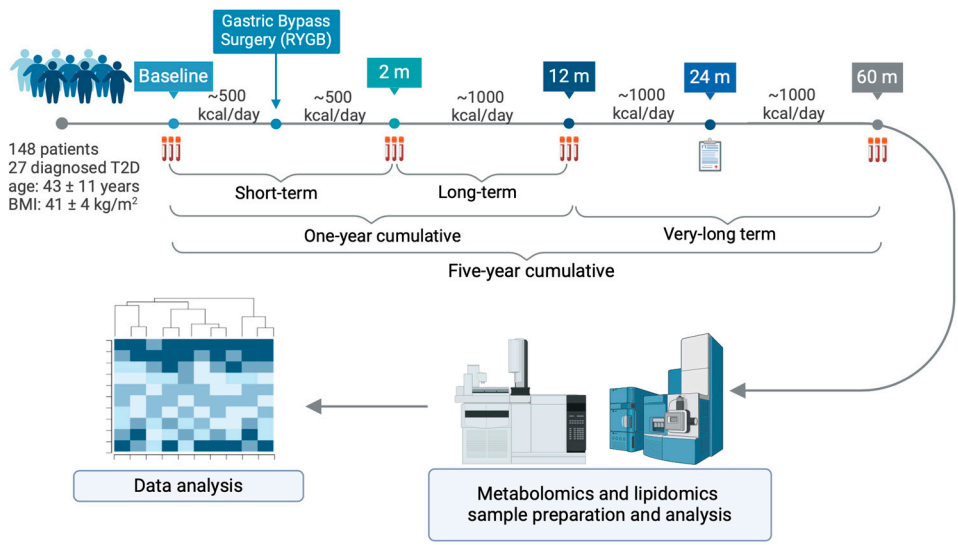


Figure 21. The outline of the study performed in Paper I.

Two types of extracts were obtained for each sample: polar extracts [96, 136] and lipophilic extracts [101, 102], which were analysed using LC-QTOF. Subsequently, fractions of the polar extracts were evaporated to dryness and analysed by GC-MS

after a two-step derivatisation involving methoximation with subsequent silylation [104].

The obtained experimental data were evaluated using PCA to get a general overview of the data (**Paper I**, **Figures 2** and **5**). Then, LMMs were applied to analyse variation in each metabolite individually over the study periods (**Paper I**, **Figures 3** and **6**). Changes in metabolite and lipid profiles were examined over the following study periods: *short-term* (from baseline to two months post-RYGB), *long-term* (between two and twelve months), *one-year cumulative* (from baseline to twelve months) and also *very-long-term* (between one and five years post-RYGB) and *five-year cumulative* (from baseline to five years) (**Figure 21**).

Analysis of the lipidomics data revealed a great diversity in the behaviour of various lipid species, but also within many of the investigated species. In order to evaluate these differences, we used OPLS-EP [126] to study lipid alterations based on their acyl chain carbon number and degree of unsaturation (**Paper I**, **Figure 4**). The loadings from the model were extracted [137], which describe the contribution of each lipid to the observed difference between study periods. Then, linear models were used to evaluate how these loadings depended on the lipid acyl chain length and degree of unsaturation. These analyses revealed that during the short period of two months post-RYGB, levels of lipids with longer acyl chains and a higher degree of unsaturation were increasing, whereas lipids with shorter acyl chains and a lower number of double bonds were decreasing. During the following ten-month post-RYGB, we observed the opposite behaviour.

The increase in long-chain and highly unsaturated lipids has been observed previously after bariatric surgeries [138, 139], although not to the same systematic extent, and are generally believed to have beneficial effects on health. Long and unsaturated lipid acyl chains contribute to enhanced membrane fluidity, which improves the capacity of the membrane to integrate proteins, including receptors and ion channels [140, 141]. The observed opposite alterations in lipid levels between two and twelve months post-RYGB (**Paper I**, **Figure 3**) demonstrate that the metabolome begins to return to its initial state. Moreover, the effect of the very-long-term follow-up showed almost no changes between the five year and baseline states (**Paper I**, **Figure S2**). Despite this observation, a complete remission of the metabolome to the initial state cannot be confirmed due to the lack of samples from the 5 years study visit.

The analysis of polar metabolites, including amino acids, acylcarnitines, carboxylic acids, sugars, and some other low molecular weight metabolites showed more complex alterations, which are summarised in (**Table 1**). Levels of the majority of metabolites decreased shortly after RYGB. These metabolites included branched-chain (leucine, isoleucine, valine), aromatic (tryptophane, phenylalanine, tyrosine), and some other amino acids (alanine, glutamic acid, lysine, ornithine, aspartic acid, proline, methionine, cysteine, threonine), sugars and their derivatives (glucose,

Table 1. Overview of alterations of metabolites between baseline and two-months post-RYGB.

Metabolites decreased in the short-term post-RYGB	Biological interpretation	References
Branched chain amino acids: leucine, isoleucine, valine	Increased levels are assosiated with insulin resistance and can be used as predictors of T2D development	[142], [143], [79]
Aromatic aminoacids: tryptophane, phenylalanine, tyrosine	Increased levels are assosiated with insulin resistance and can be used as predictors of T2D development	[79]
Other amino acids: alanine, glutamic acid, lysine, ornithine	Alanine, lysine, ornithine have been associated with T2D development	[144]
Aspartic acid, proline, methionine, cysteine	Decrease in aspartic acid, proline, methionine, cysteine reflects a catabolic state Decreased levels of methionine and cysteine are associated with oxidative stress	[145, 146]
Urea	Urea is produced in amino acid catabolism; higher levels of urea are associated with an increased risk of T2D development	[147]
Sugars: glucose, fructose, inositol	Improved insulin sensitivity	
Carnitine and short-chain acylcarnitines (3:0, 4:0, 5:0, 8:1)	Reduced catabolism of amino acids	[148]
2-Hydroxybutyrate	Levels of 2-hydroxybutyrate increase during conditions of increased oxidative stress and are associated with T2D	[149]
Lactic acid	Increased mitochondrial oxidation of glucose	[150]
Uric acid	Increased levels are assosiated with insulin resistance	[151]
Metabolites increased in the short term post RYGB		
3-Hydroxybutyrate	Ketone body, indicator of a catabolic state	[152]
Serine	Serine has been reported to be positively correlated with insulin secretion and sensitivity	[153]
Glycine	Glycine levels are decreased in patients with obesity or diabetes	[154], [155]
Acetylcarnitine (2:0), medium and long-chain acylcarnitines (10:0, 12:1, 14:1, 16:0, 18:1)	Increased levels of acylcarnitines are associated with a catabolic state	[148]
TCA cycle intermediates: malate and citrate	Possible indicators of increased mitochondrial oxidative metabolism	[156]

fructose, inositol, threonic acid), short-chain carnitines, lactic acid, 2-hydroxybutyrate, urea, and uric acid. At the same time, some metabolites increased over the short-term period, including the ketone body 3-hydroxybutyrate, serine and glycine, acetylcarnitine, medium and long-chain acylcarnitines, TCA-cycle intermediates (malic and citric acids). In general, a pattern similar to that found in the lipidomics data was observed in the metabolomics data, i.e. opposite metabolic changes over the short-term and long-term study periods, with one- and five-year cumulative effects being less significant (**Paper I**, **Figure 6**, **Figure S3**).

The majority of alterations observed are in line with previously published data on metabolite and lipid levels after bariatric surgery [157, 158]. At the same time, we found that the metabolome was shifting to the initial state two months post-RYGB. This shift parallels the expected increase in calorie intake during the post-surgery period. A few weeks prior to the surgery and until two months post-RYGB, people can consume only around 500 kcal per day. After two months, the calory intake increases to about 1000 kcal and thereafter continuous to increase [159].

The early shift toward the initial metabolome could be an early indication of future weight regain. Although gastric bypass provides significant weight loss, many patients experience weight regain in the long term. It was demonstrated that 37% of patients experience a weight regain of >25% of the total lost weight within a few years after the surgery [160]. Additionally, weight regain is associated with the recurrence of obesity-related comorbidities, such as T2D and hypertension. It has been shown that within 3-15 years after bariatric surgery 30-50% of patients who achieved T2D remission return to an insulin resistant state [161, 162].

Weight regain after bariatric surgeries could be related to anatomical changes, although this is quite rare. The most common reasons are linked to increased calory intake due to maladaptive (unhealthy eating behaviour in which food is used to cope with difficult emotions and stress) or dysregulated eating (such as grazing – eating small portions of food in an uncontrolled and unplanned way), as well as not following post-surgery dietary recommendations, insufficient physical activity, and physiological compensatory mechanism such as changes in hormonal regulation of food intake [163]. At the same time, as highlighted in the review by Busetto et al., multiple biological processes are involved in the regulation of body weight, and most of these processes are beyond voluntary food intake and physical activity [164]. Thus, the mechanisms underlying weigh regain after bariatric surgery are very complex and require further investigation.

4.2. Impact of caffeine on liver metabolism in healthy conditions – Paper II

Coffee has been suggested to cause several beneficial effects on human health, including reduced all-cause mortality, a reduction in CVDs, and positive effects on liver health [69]. Coffee has a very complex composition, and many compounds found in coffee may contribute to these beneficial effects. In **Paper II**, we focused on caffeine, since it has been shown that decaffeinated coffee lacks a significant metabolic effect in the liver [69]. The beneficial effects of coffee on liver health have been linked to its ability to improve hepatic steatosis by reducing liver fat accumulation. However, previous research has focused on the lipotoxic unhealthy liver, whereas little is known about the effect of caffeine on the healthy liver.

Initially, the effect of caffeine supplementation was evaluated in an animal model in a collaboration project with the Department of Neonatology, Lund University, where the *in vivo* experiments were performed. Several lipid species showed decreased levels in livers from caffeine treated animals at a nominal level of significance. However, these changes were minor and lost upon correction for multiple testing (**Paper II**, **Figure 2**). No statistically significant alterations in lipid profiles were found in the blood samples.

The liver is a complex organ, containing multiple cell types and *in vivo* animal research is often associated with significant variation between animals. Hence, to reduce the experimental variation and to focus on lipid metabolism in liver cells, the effect of caffeine and its metabolite paraxanthine was compared to the effect of adenosine in two independent hepatocyte cell lines. Paraxanthine was selected as it is suggested to be a more potent phosphodiesterases (PDE) inhibitor than caffeine [165]. Adenosine was included since xanthines are known to act as adenosine receptor antagonist [166]. Liver cell lines HepG2 and Huh-7 were treated with different concentrations of caffeine, paraxanthine, and adenosine, and were cultured in either low- or high-glucose media for 24h (**Figure 22**). Subsequently, lipids were extracted according to previously published method [102] and analysed using the targeted LC-QQQ method.

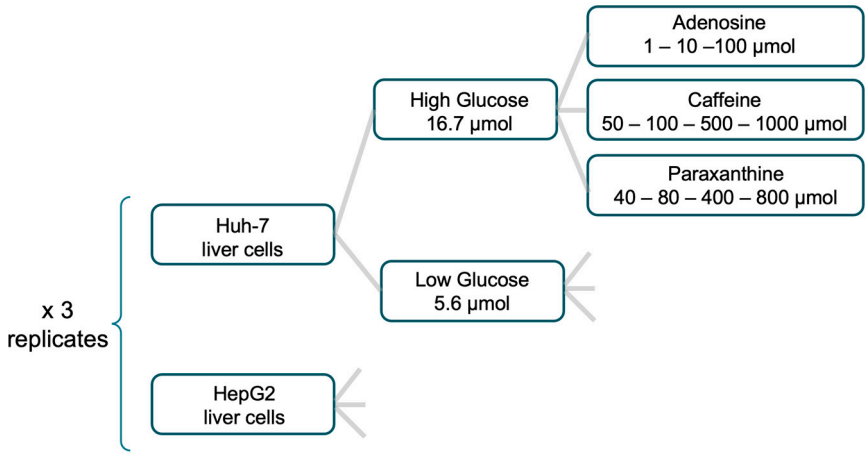


Figure 22. Schematic illustration of the setup of the experiment in lever cells performed in Paper II.

The targeted QQQ method was chosen for the lipid analysis due to the low number of cells and, consequently, the expected very low lipid abundance in the culture. The method was based on a targeted SFC-QQQ method [167]. The choice of lipid species included in the method was also based on published results [167], general information about lipid abundance in humans [6, 7, 168], and preliminary tests of a wide ranges of lipid species, from which the most abundant were selected. A summary of the selected lipids and associated transitions is provided in **Table 2** and **Figures 23-24**.

Data obtained from the cell experiments were analysed by PCA (**Paper II**, **Figure 3**). The biggest difference in the lipid profiles was found between the different cell lines and glucose concentrations, whereas no group separation was observed based on the added drugs at any of the tested concentration. The absence of effect may be explained by the fact that previous studies used higher, non-physiological, caffeine doses [169] or involved fatty acid-treated cells, which stimulated a disease state in cells [170]. In contrast, our experiment was based on "healthy" cells without the inclusion of toxic levels of fatty acids in the media. Hence, caffeine is unlikely to have an effect on the healthy liver.

Table 2. Overview of transitions for each lipid class analysed by LC-QQQ

Lipid Class	Transition	N
Positive mode ESI(+)		
PCs – Phosphatidylcholines	$[M + H]^+ \rightarrow 184$	23 + 1 IS
LPCs – Lysophosphatidylcholines	$[M + H]^+ \rightarrow 184$	15
SMs – Sphingomyelins	$[M + H]^+ \rightarrow 184$	26
Cers – Ceramides	$[M + H]^+ \rightarrow 264$	14
CEs – Cholesterol esters	$[M + NH_4]^+ \rightarrow 369$	17 + 1 IS
DGs – Diglycerides	$[M + NH_4]^+ \rightarrow [M - Acyl\text{-}COOH + H]^+$	29 + 1 IS
TGs – Triglycerides	$[M + NH_4]^+ \rightarrow [M + NH_4]^+$	39 + 1 IS
ACs – Acyl carnitines	$[M + H]^+ \rightarrow 85$	4
Negative mode ESI(-)		
FFAs - Free fatty acids	$[M - H]^- \rightarrow [M - H]^-$	24 + 1 IS
PEs - Phosphatidylethanolamines	$[M - H]^- \rightarrow [Acyl\text{-}COO]^-$	32 (53 transitions)
LPEs - Lysophosphatidylethanolamines	$[M - H]^- \rightarrow [Acyl\text{-}COO]^-$	16 + 1 IS
PIs - Phosphatidylinositols	$[M - H]^- \rightarrow 241$	11
Total		250 + 6 IS

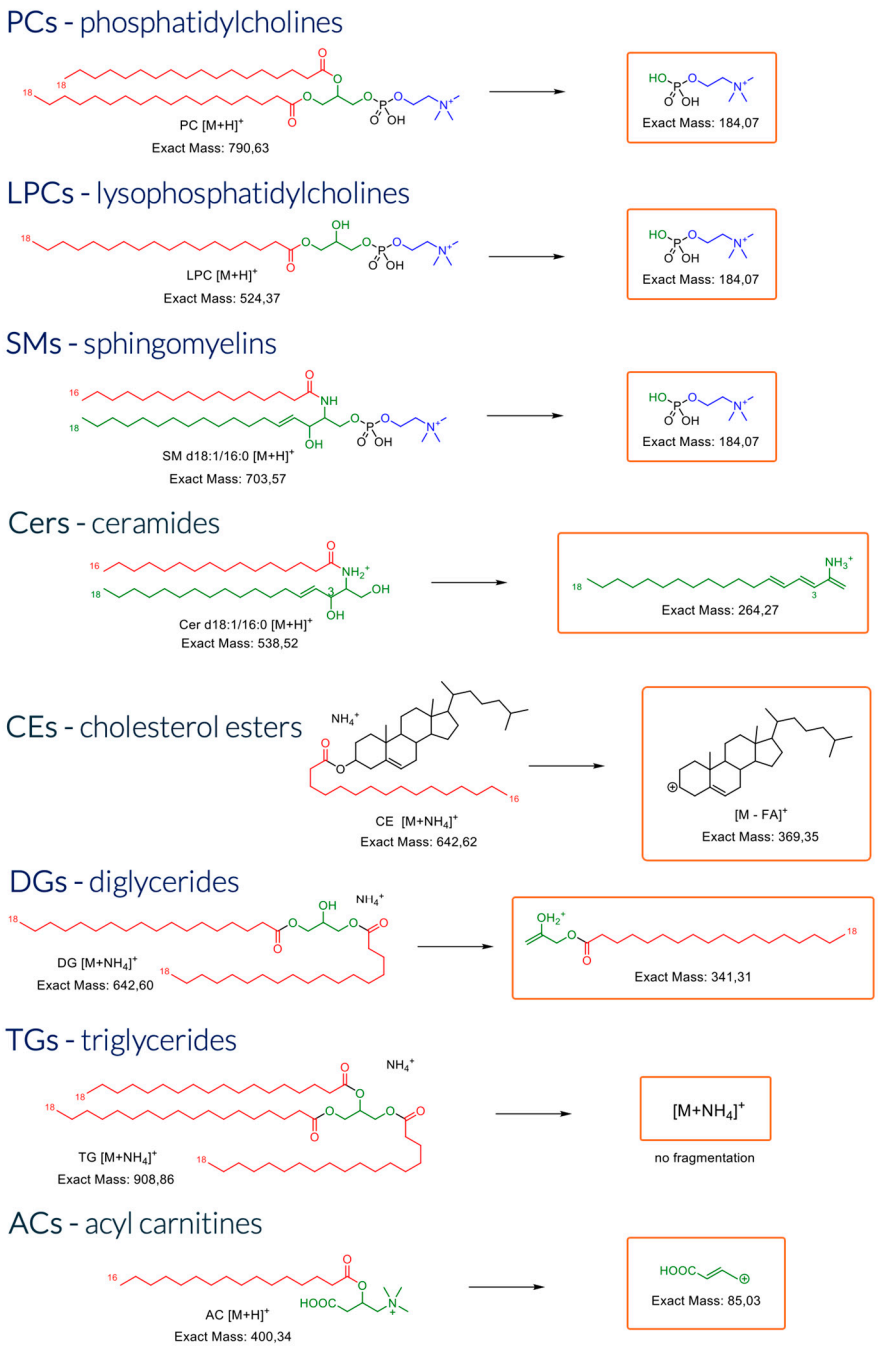


Figure 23. Examples of transition schemes for each lipid class analysed by LC-QQQ in positive ESI mode.

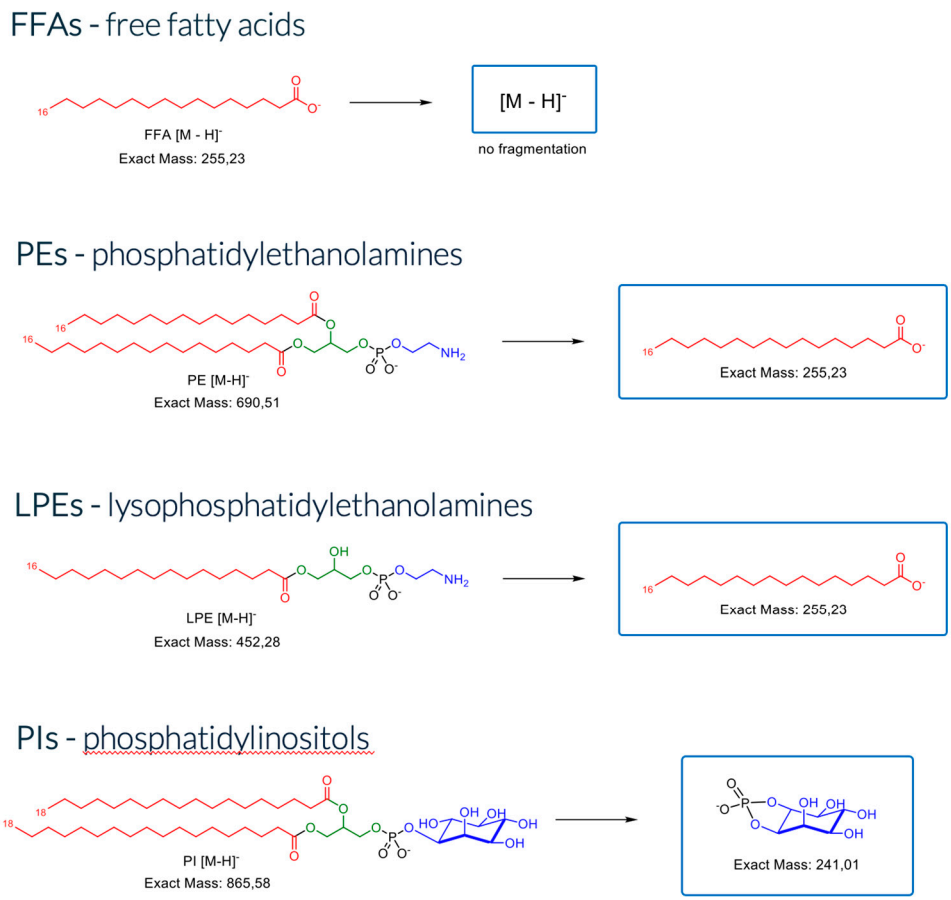


Figure 24. Examples of transition schemes for each lipid class analysed by LC-QQQ in negative ESI mode.

4.3. Impact of different dietary approaches on the metabolome of people with diabetes and normoglycemic individuals – Paper III

Diet is one of the most important factors contributing to human health and requires special attention for people with diabetes. The application of metabolomics and lipidomics approaches to evaluate the impact of different meals on health offers unique opportunities for characterising the dynamic metabolic response to varying meal composition. These meal-elicited alterations in the metabolite profile reflect a combination of endogenous and exogenous factors, where endogenous processes, influenced by genetic variation and diseases such as diabetes, interact with exogenous factors provided by the meal.

In Paper III, we evaluated the impact of four 600-kcal meals composed of red meat with boiled or French-fried potatoes and different vegetables but prepared in different ways to produce meals enriched in carbohydrates, protein, fats, or fibre. Each of these meals was given to three groups of participants: people with T1D (n = 18), people with T2D (n = 21), and a control group of individuals with normal glucose levels (n = 17), on different days with a washout period in between. Blood samples were taken five minutes before food intake and then one and three hours after the meal.

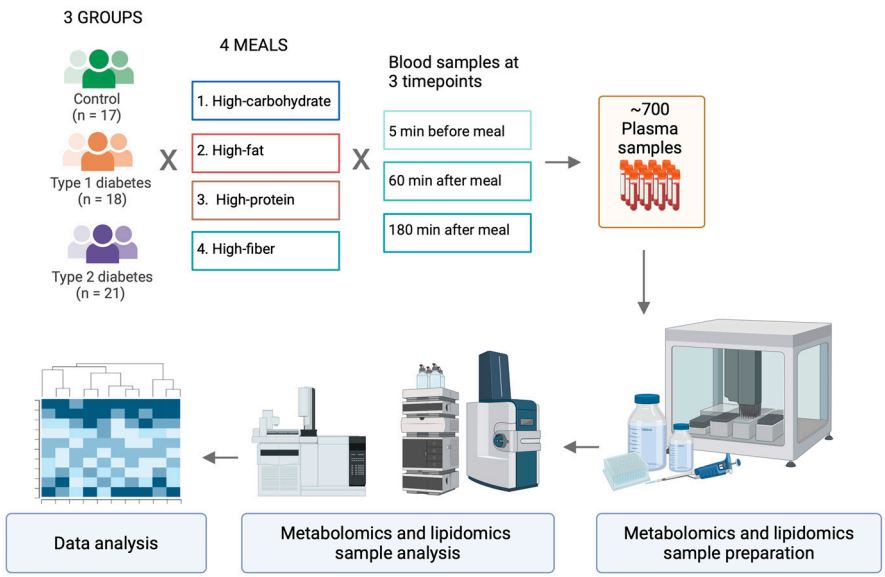


Figure 25. Overview of the study presented in Paper III.

Plasma samples were analysed by GC-MS-based metabolomics and LC-timsTOF-based lipidomics in order to evaluate the impact of meal macronutrient variation and glycaemic status on the meal-elicited metabolic regulation (**Figure 25**). Hormonal regulation in response to these meals has already been reported [171], [172].

In the obtained data, we observed that glycaemic status had a pronounced effect on lipid profiles. The group of individuals with T2D deviated from the two other groups, as evidenced by PCA (**Paper III**, **Figure 1D**), heatmaps (**Paper III**, **Figure S1**), and in analysing alterations in lipids grouped by lipid class (**Paper III**, **Figure 4**). We observed that the T2D group had higher levels of multiple lipids, mainly triglycerides (TGs) and diacylglycerols (DGs). At the same time, this group had decreased levels of lysophosphatidylcholines (LPCs), ether-linked LPCs, ether-linked phosphatidylcholines (PC-Os), and hexosylceramides (HexCer). These observations reflect a state of dyslipidaemia [173], characteristic for people with T2D, and also confirm previously published lipidomic studies in people with T2D [174, 175]. Changes in metabolite profiles based on glycaemic status were less pronounced, even though T1D group tended to show relatively lower levels of most metabolites (**Paper III**, **Figure S7**). However, some diabetes-characteristic alterations were also revealed, such as decreased levels of the hyperglycaemia-dependent metabolite 1,5-anhydroglucitol in T2D and T1D [176].

To estimate the effect of different meals on postprandial metabolite and lipid profiles, we used LMMs, which allowed us to account for the random effect associated with repeated measurements in the same individuals. In our model ($y \sim meal*time*diabetes + time^2 + (1|participant)$), we included parameters for meal, time, and diabetes status, as well as the interactions between them. We also added a squared term of time, as we observed that several metabolites and lipids showed a U-shaped profile. This model was initially used on all metabolites and lipids individually (**Paper III**, **Figures 2B-D** and **3B-D)**. We also used the same model to visualise alterations in metabolites and lipids grouped by class (**Paper III**, **Figures 4** and **6**).

Our analyses revealed several meal-dependent changes in metabolism, including an increased response in sugars following the intake of the carbohydrate- and fibre-rich meals compared to the fat- and protein-rich meals. We also found an increased level of pipecolic acid after the intake of the fibre-rich meal. Pipecolic acid is formed from lysine by intestinal bacteria and is known to increase after consumption of plants [177]. However, very few metabolites showed diabetes-modified responses, as evidenced by the lack of significant interactions in the LMMs.

Lipid levels were more sensitive to glycaemic status, as compared to the metabolites; this was mainly driven by the altered lipid levels observed in T2D. Similar results were obtained both at the level of individual lipids and at the level of lipid class. At lipid class-level, only TGs were found to be significantly altered by the meal. Notably, the TG profiles differed between saturated, mono- and

polyunsaturated TGs (**Paper III**, **Figure S5**). To examine this pattern further, we selected the most abundant lipid classes, e.g. TGs, SMs, and PCs, and evaluated how the number of double bonds and the carbon length of acyl chains were associated with the effects obtained from the LMMs (**Paper III**, **Figure 5**). We found that the protein-rich meal resulted in an increase in long-chain saturated and short-chain unsaturated PC and SM lipids, whereas it did not have a statistically significant effect on TG levels. The intake of the fat-rich meal led to an increase in long-chain saturated PCs and SMs and to an increased in medium-chain polyunsaturated TGs. As for the metabolites, we could not detect any significant interactions between diabetes and the other model terms, suggesting the meal-elicited response in lipid levels to be conserved in diabetes.

5. Conclusions and Further Perspectives

In **Paper I**, we found that the most significant alteration in the human metabolome occurs within two months after gastric bypass surgery. Then, the metabolome begins to change in the opposite direction, indicating a return to the initial state. Thus, at one-year post-RYGB, when patients reach their lowest weight, most metabolites are close to the pre-surgical level, and five years after the surgery, this difference becomes even less noticeable. The observation that metabolism returns to the initial state can provide evidence of the organism's adaptation to RYGB surgery and the safety of the procedure. On the contrary, the observed return to the initial metabolic state may indicate the reinstatement of an anabolic state, which over time will lead to weight-regain, something that is observed in the years following RYGB. Notably, not all individuals return at the same rate. Hence, it would be interesting to evaluate a longer follow-up time to test this. Moreover, the ability of metabolomics and lipidomics to predict which individuals would benefit the most from a RYGB remains unexplored.

In **Paper II**, we found that caffeine did not produce any significant changes in the lipid profiles in the *in vivo* experiment. We also did not observe significant alterations in the lipidome of hepatocytes under treatment with caffeine, paraxanthine, or adenosine. These results suggests that caffeine, in physiological concentrations, does not produce a significant impact on a healthy liver. This study, based solely on healthy animal models and clonal cell lines, does not rule out any health effects of caffeine in the obese state. However, further research is needed to more precisely identify the metabolic conditions under which caffeine may exert beneficial health effects.

In **Paper III**, we found differences in lipid and metabolite profiles depending on participants' glycaemic status and the meal consumed. However, we found very few meal-elicited alterations to be affected by glycemic status. Hence, this suggests that participants from all study groups should benefit from the same diet to the same extent. The study only evaluated the acute effects of meals. Therefore, long-term meal interventions and the assessment of additional health parameters may be necessary to validate these findings.

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Figures 1, 4, 5, 6, 14, 16, 18, 19, 20, 21, 25 were created using BioRender.

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